UTEROFERRIN: A MECHANISM OF MATERNAL TO FETAL IRON TRANSPORT IN SWINE

BY

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UTEROFERRIN: A MECHANISM OF MATERNAL TO FETAL IRON TRANSPORT IN SWINE

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Uteroferrin (UF) is an iron-containing, progesterone-induced glycoprotein with an approximate molecular weight of 35,000. This protein is present in uterine secretions of nonpregnant swine between days 12 and 16 of the estrous cycle and is also observed in allantoic fluid and uterine secretions of pregnant swine between days 30 and 100 of gestation. This study was designed to examine the role of UF in maternal to fetal iron transport.

Experiment I utilized 21 pregnant crossbred gilts which were hysterectomized either on day 30, 45, 60, 75, 90, 105 or 112 of gestation. Fetal fluids and tissues were collected and analyzed for changes in iron content and UF levels during gestation. Concentration of fetal liver (FL) and placental (PL) iron was affected by day of gestation ($P < .05$) as were total FL iron, total PL iron, allantoic fluid (ALF) iron concentration and total ALF

iron ($P < .01$). The UF content of ALF (measured by acid phosphatase activity) was affected by day ($P < .01$). Placental UF concentration was quantified by acid phosphatase activity and was affected by day ($P < .05$). Concentration and total UF content of PL, as measured by radioimmunoassay, changed during gestation ($P < .01$). A temporal relationship was observed between iron content and UF levels in both ALF and PL.

Experiment II examined effects of maternal iron treatment between days 40 and 60 of gestation on iron content and UF levels in conceptus fluids and tissues on day 90 of gestation. Eight gilts were bred and divided between two treatment groups: 1) Control--no supplemental iron injections; 2) Iron treatment--total of 22 mg iron dextran/kg body weight divided among five injections between days 40 and 60 of gestation. All gilts were hysterectomized on day 90 and fetal fluids and tissues collected. No differences were observed in iron content or UF levels in FL and ALF; however, concentration of UF in PL was higher ($P < .01$) in the iron treatment group. Total UF content of PL followed a similar trend but was not significant ($P > .05$).

In experiment III, eight gilts were bred and assigned to treatment on day 30, 60, 90 or 105 of gestation. Three gilts were rendered unilaterally pregnant and assigned to treatment on day 60 of gestation. On the assigned day, 100μCi ^{59}Fe was injected intravenously into each gilt. After 24 h, gilts were hysterectomized and fetal fluids and tissues collected.

Radiolabeled ^{59}Fe-UF was isolated from concentrated ALF and uterine flushings from day 60 unilaterally pregnant gilts. Accumulation of ^{59}Fe was also observed in fetal tissues on all days examined.

In experiment IV, *in vitro* and *in vivo* absorption of macromolecules by day 60 allantoic epithelium was examined. Absorption of fluorescein labeled γ globulin (FγG), uteroferrin (FUF) and transferrin (FTF) was observed in allantoic epithelium incubated in vitro at 37 C for 30 minutes. Twenty-four hours after introduction of FγG into allantoic fluid sacs of pregnant gilts, uptake of FγG by allantoic epithelium was detected. When γ globulin (γG) was introduced in a similar manner, γG was observed in fetal serum after 24 h.

In summary, these experiments indicate a temporal relationship between iron content and UF levels in ALF and PL. Iron treatment of the dam during peak production of UF affected ($P < .01$) placental UF content, but had no effect on fetal iron levels. Uteroferrin appears to be the major iron-containing uterine secretory product during mid-gestation and allantoic epithelium is capable of absorbing macromolecules present in ALF. Uteroferrin appears to be involved in iron transport in swine during gestation.

CHAPTER I
INTRODUCTION

During the course of gestation, the porcine uterus secretes an abundance of proteins comprising the macromolecular portion of histotrophe or uterine milk. One such protein is uteroferrin, an iron-containing, progesterone induced glycoprotein first described by Murray et al. (1972) and Chen et al. (1973). Bazer et al. (1975) reported that this protein was produced by the maternal endometrium, transported across the placenta at specific regions known as areolae and sequestered in the allantoic fluid.

Schlosnagle et al. (1974) proposed a number of possible roles for uteroferrin: maintenance of a compatible environment for the development of the embryo, hormone and vitamin transport, regulation of genetic activity due to the protein's pyrophosphatase activity and finally a role in the metabolism of iron. The last proposal, that of a role in iron metabolism, appears to be the most likely function of uteroferrin. Maternal to fetal iron transport has been examined in various species including the human (Pommerenke et al., 1942), rat (Nylander, 1953), rabbit (Bothwell et al., 1958; Baker and Morgan 1969), sheep (Hoskins and Hansard, 1964a), and swine (Hoskins and Hansard, 1964b; Palludan et al., 1969). Various means of transfer have been

proposed including phagocytosis of extravasated maternal red blood cells within hematomes or hemophagous organs of the trophoblast (Torbit et al., 1971), transport of serum iron directly across the chorioallantois (Sinha and Mossman, 1966), direct transfer of serum iron across the yolk sac placenta (Amoroso, 1952) and by uterine secretions (Wislocki and Dempsey, 1946). Seal et al. (1972) demonstrated that the rate of maternal to fetal serum iron transfer was dependent upon placental type. In species with hemochorial placentation the rate of iron transfer was 50 times greater than in those species with endotheliochorial or epitheliochorial placental types. Similar results were obtained by Baker and Morgan (1973).

The extremely low rates of iron transfer in these species can be attributed to the fact that the iron supplied to the fetus was from a source other than maternal transferrin. As previously mentioned, one possible source of iron for the developing conceptus is from uterine secretions. In species with epitheliochorial placentae, the trophoblast is separated from the maternal blood and histotrophe may play a major role in iron transport.

Interest in iron transport related to anemia prevention is especially keen in swine in which neonatal anemia is of great economic concern. Ducsay (1977) has indicated the possible role of uteroferrin in maternal to fetal iron transport in swine. When the dam was provided with additional iron during the period of peak movement of

uteroferrin across the placenta, increases in both fetal iron stores and neonatal hemoglobin concentrations were observed.

The present study was initiated to examine further the role of uteroferrin in maternal to fetal iron transport in swine. Specific emphasis was placed on the mode of iron transport, the role of the allantoic epithelium in iron uptake and uteroferrin content of the placenta. In addition, an effort was made to establish the temporal relationship between uteroferrin accumulation and the level of iron storage in the fetal tissues, fluids and membranes.

CHAPTER II
LITERATURE REVIEW

Iron

Iron has played a major role in both the geochemical and biochemical history of earth. About 35% of the earth is composed of iron (Frieden, 1974). This is in contrast to the relatively small proportion that this element comprises of the animal body; only about 0.005%. However, this small percentage does not decrease its importance. Without iron, no eucaryotes and few procaryotes are able to survive. Neilands (1972) has even indicated that procaryotes, containing only the most rudimentary iron metabolism, might be derived from the "primordial cell." This concept is based on the fact that only certain anaerobic bacteria do not contain heme compounds. Ferredoxins and rubredoxins, in which iron is coordinated with sulfur atoms or eysteine are found in anaerobic bacteria, represent the most primitive iron binding systems.

The most important biological role played by iron is in electron transfer and oxidation, i.e., respiration in the cytochrome chain. In order for this system to remain operative, an iron ligating mechanism as well as an iron transport system are required.

Heme Proteins

The cytochromes, first described by MacMunn (1885) are all heme proteins in which iron is in the ferric form in the oxidized state. Keilin (1925) recognized that the cytochromes are cellular iron-containing proteins involved in the process of electron transport. Most intracellular hemoproteins are cytochromes, with notable exception being hemoglobin and myoglobin. The structure of cytochrome c has been described by Ehrenberg and Theorell (1955) and Fitch and Margoliash (1967), in which the heme portion of the molecule is quite similar to that of hemoglobin. The iron forms a portion of the prosthetic group in the active site of the molecule. The majority of mammalian cellular respiration takes place via the mitochondrial cytochrome system (Nicholls, 1974).

Hemoglobin (Hb), which is a complex of globin and four heme moities, is essential to vertebrate life. This is due to the role of the ferrous iron of hemoglobin to combine with oxygen reversibly, thus serving as an effective carrier of oxygen (Perutz, 1979).

Zinoffsky (1886) crystallized equine hemoglobin and found it to contain 0.35% iron. Purified hemoglobin from most other mammalian species has a similar iron content (Moore and Dubach, 1962) and a molecular weight of approximately 65,000. Both the synthesis of heme and its attachment to globin occur in the bone marrow during the latter

part of red cell development. It appears that the pathway by which iron enters the erythrocyte precursor is via binding of transferrin to the erythrocyte membrane. The iron is then released and taken up by the cell, while the transferrin is recycled (Katz, 1965; Fairbanks et al., 1971). More recent studies by Trump and Berezesky (1977) indicate that the transferrin molecule may actually enter the erythrocyte before being recycled. This point, however, remains controversial.

Hemoglobin levels and total iron content of the animal body are affected by breed, age, nutrition, pregnancy, lactation, health status and environment. In human blood, there is a rapid decline from levels of 18-19g Hb/100ml at birth to 12g Hb/100ml at 3-4 months of age. After this point, there is a gradual increase to adult values of 13-15g Hb/100ml. Mean levels for other species range from 10-11 g Hb/100ml for the pig, sheep and horse, 11-12 g Hb/100ml for the rabbit and cow to 13-14 g Hb/100ml for the dog (Underwood, 1977). The mean ratio of hemoglobin to body weight was determined to be 12.7g Hb/kg in five different species (Drabkin, 1951).

Myoglobin, like all heme proteins, is involved in oxidative metabolism. The structure of this molecule is related to the single units of hemoglobin containing a single iron atom (Dallman, 1974). Myoglobin iron is about 20% of that in hemoglobin form and accounts for approximately 10% of total body iron (Åekson et al., 1968).

Although not normally depleted in the human during iron deficiency, a severe decline can be observed in chicks (Davis et al., 1968), rats (Cusak and Brown, 1965; Edgerson et al., 1972) and piglets (Gubler et al., 1957). The degree of depletion varies according to the type of muscle observed.

Non-Heme Proteins

Hemosiderin and ferritin are both non-heme compounds involved in tissue iron storage. The two proteins are slightly different in structure, but both are normally found in liver, bone marrow and spleen.

Hemosiderin is a granular, readily stainable pigment composed of ferric hydroxide and contains up to 35% iron (Shoden and Sturgeon, 1963). Granick (1945) initially proposed that hemosiderin was a breakdown product of ferritin. Analysis by Wöhler (1963) and McKay and Fineberg (1964) indicated a variety of organic components including proteins. It was also suggested that the hemosiderin granules may actually be cellular iron-containing organelles.

Ferritin, unlike hemosiderin, is a water soluble compound (Gabrio et al., 1953; Kaldor, 1958). This protein is composed of approximately 24 subunits which can sequester variable amounts of iron. Polyacrylamide gel electrophoresis (Linder-Horowitz et al., 1970), isoelectric focusing (Arosio et al., 1976) as well as immunological techniques (Linder and Munro, 1973) indicate that ferritin

occurs in different forms. These forms occur both in different tissues as well as within the same tissue. The apo (iron-free) form of ferritin has a molecular weight of approximately 460,000 (Harrison et al., 1974). The synthesis of ferritin protein is stimulated by iron in various cell types. A specific response to increased extracellular concentrations of iron occurs either at the posttranscriptional (Zähringer et al., 1977) or posttranslational level (Drysdale and Munro, 1966; Munro and Drysdale, 1970).

The relationship between ferritin and hemosiderin and the factors affecting the relative proportion of each in the liver and spleen were examined in rabbits (Schoden et al., 1953) and humans (Morgan and Walters, 1963). Iron appeared to be utilized at the same rate from both compounds. Deposition of iron was equally distributed. In disease states characterized by very high tissue iron levels, hemosiderin appears to be the major tissue storage form of iron.

In studies with neonatal pigs, it was determined that the ratio of ferritin to hemosiderin iron was about 2 to 1 (Furugouri, 1973). During the period of rapid postnatal growth, however, in which depletion of iron occurs, the ratio declined to about 1 to 6. These findings are in agreement with earlier work with the rat (Mazur and Carelton, 1965) which indicated that ferritin was mobilized more rapidly than hemosiderin.

As described previously, the liver and spleen are the major sites of iron storage. In studies by Furugouri (1973),

however, no major changes in the ferritin-hemosiderin ratio in the spleen of piglets under study were observed. This organ is a major site of red cell destruction and is therefore more important in this capacity than in iron storage (Leslie and Kaldor, 1971).

The third major non-heme iron compound is transferrin, which is the principal iron transport protein in the blood of mammals. This protein has a molecular weight which has been estimated between 76,000 to 80,000 and two active sites for iron, i.e., it binds two iron atoms per molecule (Ehrenberg and Laurell, 1955; Greene and Feeney, 1968; Sly and Bezkorovainy, 1974). Fletcher and Huens (1967, 1968) proposed that these two binding sites were biologically different and electron paramagnetic spectroscopy studies have provided support for this concept (Aisen et al., 1969; Price and Gibson, 1972).

Serum iron levels range from 63-202 mg/100ml of which most, if not all, of the iron is bound to transferrin (Schade et al., 1949). Prasad and Oberleas (1971) found after incubation of pooled human serum in vitro with ^{59}Fe that ultrafilterable iron was 5-7.5% of the total serum iron. This difference was due to iron binding by albumin and amino acids. The normal level of iron saturation of transferrin is between 30 and 40%. The remaining portion is referred to as the latent iron-binding capacity, while the sum of the two is known as the total iron-binding capacity (Schade et al., 1954; Underwood, 1977). If serum

iron levels fall as in iron definciency anemia or during late pregnancy, the total iron-binding capacity (TIBC) increases while the percent saturation declines. Serum iron and TIBC levels have also been determined in the large domestic species. Studies in pigs (Furugouri, 1971; 1972), sheep (Underwood and Morgan, 1963; Gardiner, 1965) and cattle (Underwood and Morgan, 1963) indicate serum iron levels are higher than those found in humans, but little differences among the species examined were observed.

Lactoferrin is another non-heme protein that bears a striking resemblance to transferrin, especially in its iron-binding capacity. The presence of this protein was first demonstrated in bovine milk (Sörensen and Sörensen, 1939). A similar ferroprotein was also found in human milk (Johannson, 1960; Montreuil et al., 1960). The name lactoferrin is, however, misleading. Although it was first isolated from milk, lactoferrin has since been isolated from saliva and pancreatic fluids (Masson and Heremans, 1966a), bile (van Vugt et al., 1975), semen (Roberts and Boursnell, 1975), lacrimal fluid (Broekhuyse, 1976) and uterine secretions (Dixon and Gibbons, 1979).

The molecular weight of lactoferrin is approximately 77,000, and it consists of a single peptide chain (Castellino et al., 1970; Querinjean et al., 1971). Like transferrin, this protein can bind two iron atoms but is normally only partially saturated. Additionally, the optical spectra of the two proteins are quite similar. The lactoferrin-iron

complex, however, is more stable at low pH than the transferrin-iron complex. Differences also occur relative to antigenicity and amino acid composition (Masson and Heremans, 1966b; Brown and Parry, 1974).

Masson et al. (1968) identified lactoferrin in the gastrointestinal tract of guinea pigs and humans. In vitro experiments with exogenous apolactoferrin indicated that this protein inhibited accumulation of iron in the intestinal wall but did not block net iron transfer to the serosal surface. Similar data by van Vugt et al. (1975) indicated that lactoferrin may play a regulatory role in iron absorption; specifically, a protective function when increased iron absorption is necessary or when excess iron intake occurs. A bacteriostatic effect of lactoferrin has also been demonstrated (Masson and Heremans, 1966a; Bullen, 1976).

Iron Absorption

Due to the limited ability of the body to excrete iron, the principal area for control is absorption. Iron absorption at the mucosal level occurs in two steps: 1) mucosal uptake of iron from the lumen and 2) mucosal cell transfer of this iron across the serosal membrane to the circulation (Manis and Schacter, 1962). Early studies by McCance and Widdowson (1937, 1938) demonstrated that absorption of iron is controlled by body requirements. Chodos and Ross (1953) later supported this concept utilizing tracer studies. Hahn

et al. (1943) postulated the "mucosal block" concept of iron absorption. This theory, further advanced by Granick (1946), proposes that the intestinal mucosa absorbs iron according to levels found within the cells. Iron absorbed by the mucosal cells is incorporated into ferritin, and when saturation occurs, no further iron uptake takes place. Once the iron is released from ferritin and transferred to plasma via transferrin, iron absorption is resumed. Conrad and Crosby (1963) in later studies with rats determined that regulation of iron absorption was dependent upon the iron concentration in the mucosal epithelium. However, as Prasad (1978) points out, various experiments have negated the idea of a "mucosal block": 1) in the human, increased absorption of iron occurs after increased doses of iron; 2) species differences are observed in examining mucosal ferritin content after oral iron administration; and 3) in guinea pigs, the peak in plasma iron levels precedes the maximal accumulation of ferritin in the intestinal mucosa after an oral iron dose, the converse of the mechanism of the "mucosal block."

Although no definitive identification has been made, it appears that specific iron-binding proteins play an integral role in the movement of iron from the intestinal lumen, across the intestine and into the circulation. Greenberger and Ruppert (1966) utilized cycloheximide, an inhibitor of protein synthesis, in rats and observed an inhibition of iron uptake. This suggested that the inhibition was due to

a lack of an unidentified carrier substance. Manis (1971) also found an impairment in iron transport at the intestinal level of mutant mice with sex-linked anemia. This defect was attributed to lack of protein production at the level of the intestine. After injecting rats with ^{59}Fe, Evans and Grace (1974) demonstrated that the majority of the isotope found in the intestinal mucosa was bound to the plasma membrane of the epithelial cells. It was also determined that labeled plasma membranes incubated in a transferrin or plasma-containing medium released a much greater quantity of the ^{59}Fe than membranes incubated in a control medium. The author suggested that transferrin interacts with iron receptor sites on the plasma membrane of the epithelial cells. Savin and Cook (1978) determined that iron uptake occurs by passive diffusion in isolated rat duodenal mucosal cells. This initial uptake is not affected by iron status and appears to be more a function of the bioavailability of iron in the medium. This mechanism may serve to concentrate dilute luminal iron at the mucosal surface in preparation for absorption.

Several studies have suggested the presence and activity of a transferrin-like iron-binding protein in mucosal cells involved in iron uptake and regulation. Heubers (1975) has demonstrated two iron-binding protein fractions isolated from the duodenal mucosa of rats. The first protein was identified as mucosal ferritin but was not implicated in rapid transfer of iron across the epithelial layer.

However, a second protein, designated as protein 2, appeared to have a much more rapid iron turnover rate. Turnover rate also correlated well with the rate of iron absorption, and a significant increase in this protein was observed during iron deficiency. This protein, however, did not cross-react immunologically with transferrin. Pollack and Lasky (1977) isolated a Gut Iron Binding Protein (GIBP) which had similar properties to protein 2 isolated by Heubers (1975). This protein has also been isolated from guinea pig brain, heart and spleen. This wide tissue distribution implies a role in the metabolism of ferritin or heme. Additional studies by Furugouri and Kawabata (1976) demonstrated the existence of numerous iron-binding compounds of the intestinal cell membrane of the pig.

Iron Deficiency

Numerous conditions may result in anemia, but indeed the most common form of anemia is due to iron deficiency. Moore (1958) indicated that iron deficiency may occur due to excessive blood loss, i.e., hemorrhage, inadequate iron absorption or a compounding of the two problems. Iron deficiency may also occur in the neonate due to inadequate placental transport of iron during the course of gestation. Three stages of iron deficiency can be defined. The first and mildest stage is the exhaustion of storage iron with no other abnormalities identifiable. Latent iron deficiency is the second stage and occurs after depletion of storage

iron. Reduced serum iron and increased transferrin concentration as well as a decline in transferrin iron saturation are observed while hemoglobin levels remain relatively normal. The third and final stage is reached when hemoglobin levels decline due to a decrease in hemoglobin synthesis.

Iron deficiency anemia is a well documented disorder in human populations (McCance and Widdowson, 1951; Hill and Starcher, 1965; Banerji et al., 1968). Under normal conditions, however, it is not a naturally occurring phenomenon in most other mammalian species including the large domestic species. Calves, foals and lambs possess comparatively large iron stores at birth. Hibbs et al. (1963) have also indicated that these species begin to consume appreciable quantities of feed at an early age and are therefore not as dependent upon milk as an iron source. Swine, however, are an exception to this concept. Neonatal swine are quite susceptible to iron deficiency anemia, especially prior to weaning.

Miller (1977) outlined four basic factors involved in the development of iron deficiency anemia in the nursing pig: 1) indoor confinement and consequential loss of contact with soil; 2) extremely rapid rate of growth; 3) inadequate iron content of porcine colostrum and milk; and 4) low body content and storage of iron at birth. In essence, the basic problem is man-made. With increased selection for rapid growth rate and the trend toward large, indoor operations, the problem of piglet iron deficiency has been intensified.

The problem was initially recognized by McGowen and Crichton (1923) and Doyle et al. (1927). They observed that iron deficiency anemia was more prevalent among swine raised in confinement than among those animals having access to soil. Later studies by Venn et al. (1947) indicated that nursing piglets with free access to soil contained large amounts of soil in their intestinal tract. The iron content of the soil was approximately 1.4%, again emphasizing the importance of contact with soil in supplying iron to the nursing animal.

As mentioned previously, the rapid growth rate of the nursing pig is another major contributing factor to iron deficiency. By the end of six weeks the pig has increased its birthweight by 1000%. This is in marked contrast to the lamb with a 400% increase and the calf and human infant with only a 200% increase in preweaning growth rate (Miller, 1977). The rapid rate of growth of the pig places incredible demands on its meager iron stores. Compounding this problem is the inadequate iron content of swine milk. Milk is a relatively poor iron source. Human milk contains approximately 0.5 mg iron/liter (Cavell and Widdowson, 1964) with similar levels observed in bovine milk (Feuillen and Plumier, 1952). Pond et al. (1965) estimated comparatively higher levels for swine milk ranging from 1.4 to 3.4 mg/liter. These levels, however, are quite inadequate to supply the demand of the rapidly growing piglet. Venn et al. (1947) estimated that the nursing piglet obtains about 1 mg iron/day

from suckling, but the growing piglet requires almost 7 mg of iron/day during the suckling period.

Various methods of iron therapy have been attempted to overcome the deficiency of this essential nutrient during the suckling period. Oral iron therapy ranging from the use of ferric oxide (Pickett et al., 1961), iron carbonate and ferrous sulfate (Harmon et al., 1967) has been attempted with ferrous sulfate being the most effective compound. Up to 12 hours postpartum, orally administered iron-dextran is also readily available for uptake and incorporation into hemoglobin synthesis (Furugouri and Kawabata, 1975; Thoren-Tolling, 1975).

At present, the most successful form of iron therapy is the parenteral administration of iron-dextran. The normal procedure is to inject 100mg of iron dextran intramuscularly two to three days after birth. The amount of iron supplied is sufficient to maintain adequate hemoglobin levels throughout the critical suckling period (Rydberg et al., 1959; Braude et al., 1962; Miller et al., 1973). In an effort to augment the small iron stores of the piglet at birth various workers have attempted prefarrowing iron treatment of the dam (Rydberg et al., 1959; Pond et al., 1961). No positive effects were observed when sows were injected with iron-dextran up to four weeks prior to parturition. Ducsay (1977) noted an increase in fetal iron stores and neonatal hemoglobin in piglets from sows treated much earlier in gestation. The iron treatment was given

during the time when the iron containing protein uteroferrin was reaching maximal levels (between days 40 and 60 of gestation). The increased iron levels were however insufficient to obviate the need for supplemental iron.

Placental Iron Transport

In order to understand more fully the reason for the low body stores of iron in the neonatal pig, one must first examine the anatomy and physiology of placental transfer.

The mammalian placenta has been a source of great curiosity, mystery, abstract guesses and intense study since ancient times. Some of the earliest recorded work was from Galen in which he described both the structure and function of the fetal membranes of the goat (Steven, 1975). Although Galen's concepts were based on intense observation, his theories were not always based objectively on his studies. His concepts however remained dogma until the dawn of the Renaissance when men like Leonardo da Vinci, Vesalius and Fabricius seriously questioned earlier teachings. An outpouring of observations and drawings continued throughout the 17^{th}, 18^{th} and 19^{th} centuries ranging from Walter Needham's work in the area of embryology to the descriptive work of Turner in 1876 (Steven, 1975) who described the state of knowledge of the placenta:

> . . . the fetal placenta possesses an absorbing surface; the maternal placenta a secreting surface. The foetus is a parasite which is nourished by the juices of the mother. . . . (p. 21)

In the early portion of the 20th century Grosser proposed a histological classification of placental types (Amoroso, 1952). This system was based on the number of maternal and fetal tissue layers separating the two circulating systems:

1) Epitheliochorial- (horse, pig) — 3 maternal and 3 fetal tissue layers.
2) Syndesmochorial- (ruminants) — 2 maternal and 3 fetal layers.
3) Endotheliochorial- (carnivores) — 1 maternal and 3 fetal layers.
4) Hemochorial — only 3 fetal layers.

This categorization was quite useful but misleading, for it implied that placental permeability was directly related to the number of tissue layers present. Although this may be true in the case of simple diffusion, other factors must also be considered. Steven (1975) indicated that active transport by placental tissues, the pattern of capillary blood flow, as well as the metabolic rate of the tissue are all factors intimately involved in regulating the "placental barrier."

The fetus is dependent upon the maternal system for iron as well as a vast array of nutrients required for normal development. As Nylander (1953) indicated, the fetus is almost "parasitic" on the maternal organism. This relates to the earlier concepts of Hammond (1944) which related nutrient priority or "partitioning of nutrients" to the metabolic rate of the various tissues. Aside from the maternal brain tissue, the fetus has priority for the major

nutrients during pregnancy. Actually the fetus is "parasitic" to a limited degree. A major alteration in maternal levels of a particular nutrient (i.e., a deficiency) is observed only if maternal stores decline beyond a certain point. At this stage, placental nutrient transfer and therefore fetal nutrition will be hindered resulting in smaller offspring or in the extreme case deformed or dead fetuses (Giroud, 1968; Palludan et al., 1969). If normal maternal nutrient levels are adequately maintained, however, normal offspring are the end result.

Studies in man (Pommerenke et al., 1942; Fletcher and Sutter, 1969) and various laboratory animals (Bothwell et al., 1958; Morgan, 1964; Kaufman and Wyllie, 1970) have indicated that the major accumulation of iron in fetal tissues occurs in the latter portion of gestation. In the large domestic species similar results have been observed (Moustgaard, 1969; Hoskins and Hansard, 1964b). However, these data do not provide an explanation for the mechanisms involved and the differences observed among the various placental types.

Hemochorial placentae

This group includes humans, subhuman primates and laboratory species in which the trophoblast layer of the placenta is bathed by maternal blood. Early theories regarding placental iron transfer in these species envisioned maternal red blood cells as the major source of fetal iron.

Stander (1941) believed that the erythrocytes were either degraded or phagocytized by the trophoblast. Later studies by Pommerenke et al. (1942) argued against this concept. Studies in which oral radio-labeled iron was fed to mothers prior to delivery indicated that the label was present within the fetal circulation by 40 minutes after the initial dose was administered. This was too brief a period of time for the iron to be incorporated into eyrthrocytes and subsequently released and taken up by the fetus. There was obviously another mechanism involved. The discovery of transferrin (Holmberg and Laurell, 1947) led to experiments indicating the involvement of this iron-binding protein in iron transport from the maternal plasma to the fetal circulation.

In a series of experiments utilizing the guinea pig, Vosburgh and Flexner (1950) determined that maternal plasma iron could supply fetuses with all of the iron required for normal development. In these studies maternal plasma was labeled with ^{59}Fe and reinjected into pregnant guinea pigs in multiple doses. Their conclusions were based on the rate of disappearance of the label from the maternal circulation and the amount of ^{59}Fe measured in the fetuses. There was no evidence of breakdown of maternal erythrocytes.

Transferrin has also been shown to be taken up by the rat placenta in vitro (Laurell and Morgan, 1964) as well as in vivo (Glasser et al., 1966; Kaufman and Wyllie, 1970). The use of metabolic inhibitors has also been examined and

no effect was observed on iron uptake by placental slices (Wyllie and Kaufman, 1974). Similar uptake and release of transferrin was also observed in rabbit placenta (Baker and Morgan, 1969; McLaurin and Cotter, 1970). As time after injection increased, a decline in the amount of labeled transferrin bound by the placenta was observed. Although some transferrin crosses the placenta (Baker and Morgan, 1970), the majority of the iron transferred is not transferrin bound. Baker and Morgan (1969) also demonstrated that the flow of iron is undirectional, i.e., from the maternal to fetal system, with little or no retrograde transfer. When placentae were left attached to the uterus after removal of the fetuses, the amount of iron accumulated and the rate of transfer was found to be similar to that for the intact feto-placental unit. This demonstrates the active role the placenta plays in iron uptake.

Studies in the human have also demonstrated transferrin binding to the placenta (Fletcher and Sutter, 1969). The placenta of the human has also been shown to bind transferrin *in vitro* (Galbraith *et al.*, 1978). More recent studies (Faulk and Galbraith, 1979; Wada *et al.*, 1979) have indicated the presence of specific transferrin receptors on human trophoblast. Fluorochrome-labeled transferrin was found to bind to trophoblast, but only after washing the tissue for prolonged periods or after ammonium thiocyanate treatment. This treatment was believed to be necessary to make the binding sites available (Faulk and Galbraith,

1979). More direct evidence for placental transferrin receptors was presented by Wada et al. (1979). A membrane vesicle preparation was used in conjunction with ^{125}I-transferrin to study equilibrium binding. These studies revealed the presence of a saturable, high affinity binding site with a Ka of $3.6 \pm 1.5 \times 10^{7}\ m^{-1}$. A similar receptor may be present on rabbit placentae. This would explain the time dependent decline in amount of labeled transferrin bound observed by Baker and Morgan (1970).

The mechanism of iron transport by the placenta after transferrin binding is unclear. However, many similarities between transferrin binding to placentae and to reticulocytes have been observed. In in vitro studies, transferrin binding by the placenta (Laurell and Morgan, 1964; Wada et al., 1979) and by reticulocytes (Morgan, 1964; Wong and Morgan, 1974) has been observed. The same authors observed increased transferrin uptake from the medium by both reticulocytes and placenta. With these similarities in mind, Faulk and Galbraith (1979) proposed that the transferrin-iron complex once bound by placental receptors is then endocytosed in a manner similar to iron uptake by reticulocytes observed by Hemmaplardh and Morgan (1977). This would not be consistent with earlier work by Gitlin et al. (1964) and Baker and Morgan (1970) who found that very little of the maternal transferrin actually reached the fetal circulation. However, after endocytosis, transferrin may still be recirculated and not reach the fetal bloodstream. The release of

iron at the placental level may involve carbonate anion removal with a subsequent oxidation-reduction reaction (Aisen and Brown, 1977). Once the iron is released, it may be bound by placental ferritin (Nylander, 1953; Wohler, 1963) or a low molecular weight iron-carrier proposed by Larkin et al. (1970).

Endotheliochorial placentae

This group is represented principally by the carnivores. In this type of placentation, there is an additional tissue layer, the maternal endothelium, separating the maternal and fetal circulation. This group has not been studied as extensively as the previously described hemochorial group. Anderson (1969) indicated that in the dog in late gestation another layer, an interstitial membrane, increases in thickness as gestation progresses. However, it was also noted that the chorion becomes interspersed by fetal capillaries which come quite close to the maternal circulation, thus reducing the thickness of the "placental barrier." This fact reemphasizes the point that it is not the actual number of tissue layers separating the two blood streams, but of greater importance are the functional aspects of these layers and related structures which are involved in reducing the efficiency of placental transport.

The rate of serum-iron transfer in carnivores is much slower than in species with hemochorial placentae (Seal et al., 1972). The actual amount transferred over time is

much too low to account for the amount of iron found in the neonate at birth. There must then be another mechanism involved in the transport of iron from the maternal to the fetal system.

Based on studies in the cat and other carnivores, it appears that iron transfer occurs principally through extravasation of maternal erythrocytes into the uterine lumen. This is followed by phagocytosis by the chorionic epithelial cells and subsequent transfer of iron released from hemoglobin to the fetal capillaries (Wong and Morgan, 1974). In their study, maternal erythrocytes were labeled with ^{59}Fe and returned to the maternal circulation. The rate of iron transfer was measured and found to be approximately 176µg/day while iron transfer from plasma was only 7.4µg/day. The rate of iron transfer from erythrocytes, if maintained throughout gestation, would be adequate to account for all the iron accumulated by the fetus at term. These studies confirm the results of early work of Wislocki and Dempsey (1946), Dempsey and Wislocki (1955) and Wynn and Corbett (1969). Utilizing light and electron microscopy, these authors demonstrated that extravasated maternal erythrocytes were present in the uterine lumen, specifically in the paraplacental area of the fetal membranes. The erythrocytes were then taken up by phagocytosis by the epithelial cells of the chorion and a portion of the iron was converted to a non-heme form. Torbit et al. (1971) found that in the pregnant bitch, uterine macrophages were involved in iron

transport to the preimplanted conceptus. The iron-bearing macrophages were observed to accumulate in the endometrial stroma followed by movement to the uterine glands and subsequent discharge of iron.

Another characteristic of endothelial placentae of carnivores is the presence of effusions of maternal blood which separate the trophoblast and maternal tissues. The chorionic epithelium is in close contact with these effusions and is believed to be involved in ingestion of maternal red blood cells (Amoroso, 1952; Creed and Biggers, 1964). Creed and Biggers (1963) applied the term "hemophagous organ" to these areas. These structures lend further credence to the concept of extravasation of maternal erythrocytes for iron transport in carnivores.

Epitheliochorial placentae

The domestic ungulates as well as the Cetacea (whales, dolphins) all have epitheliochorial placentae. Under Grosser's original classification scheme, cattle and sheep were classified as syndesmochorial, but ultrastructural studies by Lawn et al. (1969) indicated that these placentae were also of the epitheliochorial type. This placental type was believed to be the "strongest barrier" to transport. Although species' differences are observed, there are a minimum of three fetal tissue layers and three maternal tissue layers separating the two circulations. One must again consider, however, how many of these layers

are actually involved in forming the "barrier" and how the conditions for exchange are altered both anatomically and metabolically as gestation progresses. In the pig, for example, during the early portion of gestation the distance between the maternal and fetal capillary beds is approximately 130 to 150μ. However, near term this distance is reduced to as little as 2 to 5μ (Palludan et al., 1969; Tiedman, 1979).

In examining iron transport, the epitheliochorial placenta does exhibit slower rates of transfer of this vital metal. In a comparative study, Seal et al. (1972) found that the rate of placental iron transport in the pig, sheep and horse was quite similar. Values ranged from .01 to .002% of the injected dose of ^{59}Fe being found in the placenta by 3 hours. The same rate was observed in species with endotheliochorial placentae. However, 6 to 48% of the injected dose was transferred to the placentae of species with hemochorial placentae. Along with studies of carnivores, data on domestic ungulates indicate a different mode of iron transport. As previously mentioned, the process of iron transport has been studied in a variety of domestic ungulates including the pig, sheep and horse. The pig has been most extensively studied of this group due to the prevalence of anemia in the neonate.

Hoskins and Hansard (1964b) examined maternal to fetal iron transport in swine as well as the distribution of labeled iron in fetal tissue. The isotope ^{59}Fe was injected

into pregnant gilts on day 35, 70, or 105 and fetal tissues were collected 48 h after injection. The total amount of ^{59}Fe absorbed by the products of conception increased throughout gestation; however, whole body specific activity of the fetuses declined between days 35 and 70. This is indicative of rapid iron incorporation and utilization during the initial portion of gestation.

Palludan et al. (1969) examined placental iron transport in pregnant gilts between days 85 and 95 of gestation. Fetuses were removed at different times after injection and in as little as 3 h, labeled iron was detectable in fetal plasma. A rapid increase in activity of the placental iron pool was also observed. This is similar to data obtained in species with hemochorial placentae (Bothwell et al., 1958; Baker and Morgan, 1970). In these species the iron transfer occurs via the hemotrophic route, i.e., by a transfer across the epithelial layer from the maternal capillaries to the trophoblast layer of the placenta. However, in the pig, it is assumed that the normal mode of nutrient transfer is via the embryotrophic route, that is by secretions from the uterine glands.

In order to examine this possibility, Palludan et al. (1969) labeled the circulating iron pool of a pregnant gilt with ^{55}Fe labeled plasma. Approximately 8 h after injection, sections of the intact uterine/placental unit were taken and prepared for histologic and autoradiographic examination. It was observed that the highest density of

radioactive granules was in cells of the uterine glands and their secretions and in areolae associated with uterine glands. This observation indicated the significant embryotrophic route of maternal to fetal iron transport in pigs.

To examine this concept further, another pregnant gilt was injected with ^{59}transferrin. In this experiment, placentae were removed 3 h after injection, and areolae as well as interareolar chorioallantoic tissue were punched out and counted. The major portion of radioactivity was found in areolae. These data are consistent with earlier findings (Wislocki and Dempsey, 1946) which demonstrated, by histochemical techniques, the presence of iron and acid phosphatase in the glandular epithelium, uterine lumen and the areolae.

Palludan et al. (1969) postulated that the trophoblast layer might be comprised of two iron pools. One pool had a high turnover rate and rapid exchange of iron within the interplacental space. The other iron pool would have a low turnover rate and the iron would be liberated as needed by the fetus. This concept would help to explain earlier findings (Glasser et al., 1966) that isolated placentae incorporate as much iron as intact feto-placental units. It would also lend some explanation to the delay noted by Palludan et al. (1969) in the time between iron uptake by the placenta and the appearance of the label in the fetal circulation.

Uterine Protein Secretions

Based on data from Wislocki and Dempsey (1946) and Palludan et al. (1969), it appears that iron transport in swine is related to uterine secretions, i.e., the embryotropic route of nutrient transfer. Comprising the environment of the early mammalian embryo, uterine secretions play a major role in their development. In domestic species, where placentation is noninvasive, secretions of the maternal endometrium are elevated to an even greater level of significance.

Bonnet (1882) indicated that the nutrient role played by uterine secretions was initially recognized by Aristotle (384-322 B.C.) and further elaborated on by William Harvey (1578-1657 A.D.). According to Amoroso (1952), the term "uterine milk" was introduced by Needham in 1667 to describe the nutritional role played by uterine secretions. Bonnet (1882) determined that uterine milk from cattle and sheep had a protein content of approximately 10%.

The composition as well as changes in the pattern of uterine protein secretion has been studied in a wide range of species: rat (Kunitake et al., 1965; Surani, 1976), mouse (Aitken, 1977; Fishel, 1979), man (Beier et al., 1970; Voss and Beato, 1977; Aitken, 1979), cow (Laster, 1977; Dixon and Gibbons, 1979), sheep (Roberts et al., 1976b) and horse Zavy, 1979). In these studies, however, little effort was made to ascribe specific roles for these proteins aside from a general nutritive function. In addition, the majority

of these proteins have not been demonstrated to be uterine specific.

Two of the most extensively studied uterine secretory proteins are uteroglobin, isolated from the rabbit uterus (Beier, 1968, 1973, 1978; Krishnan and Daniel, 1967; Bullock, 1977) and a purple phosphatase (Murray et al., 1972; Chen et al., 1973, Bazer et al., 1975). The latter protein has recently been named uteroferrin (Buhi et al., 1979).

Uteroglobin

Uteroglobin comprises the major protein fraction from rabbit uterine flushings from the period after ovulation until implantation (Beier, 1968, 1973). Polyacrylamide gel electrophoresis as well as immunodiffusion tests reveal that a number of the proteins found in uterine secretions are in fact serum proteins. Uteroglobin, however, does not originate as a transudate of serum. Of interest is the fact that a uteroglobin-like protein has been identified in rabbit bronchial secretions as well as in seminal plasma (Beier et al., 1975; Noske and Feigelson, 1976; Beier et al., 1978). Uteroglobin does not, therefore, appear to be uterine specific as initially postulated (Beier and Beier-Hellwig, 1973).

The synthesis of uteroglobin is activated principally by progesterone; however, it is the proper ratio of progesterone to estrogen that is responsible for normal uteroglobin synthesis and release into the uterin lumen (Beier,

1968; Beier, 1970). If estradiol 17B is injected 6 and 30 h post coitum, an obvious delay of 2-5 days in the uterine protein secretory pattern is observed. Such an asynchronous protein secretory pattern provides an unfavorable environment for development of blastocysts (Beier, 1970; Beier, 1974).

One major function ascribed to uteroglobin is that of a progesterone-binding protein (Beato and Beier, 1975; Beato, 1977). Kirchner (1972) also indicated the presence of this protein within blastocoelic fluid. Uteroglobin may transport progesterone directly into the blastocyst (Seamark and Lutwak-Mann, 1972) where the steroid hormone may be used in regulating the metabolic activity of the blastocyst. Uteroglobin has also been demonstrated to inhibit trypsin activity in a manner analogous to a protease inhibitor (Beier, 1976). This suggests a role in regulation of proteolytic activity at various sites in the blastocyst, particularly those which control the structural metabolism of the covering of the blastocyst itself.

Although not unique to uterine secretions, a number of other enzymes have been demonstrated. Lutwak-Mann (1955) and Böving (1965) have indicated the presence of carbonic anhydrase in rabbit endometrium, and, like uteroglobin, it was progesterone modulated. Leucine aminopeptidase (LAP) has also been isolated in the rabbit endometrium, reaching peak activity on day 6 after mating (Beier, 1971). This membrane bound enzyme has also been reported to be under progesterone control (Beier, 1974).

Uteroferrin

Progesterone appears to be intimately involved in regulating uterine protein secretions of the pig. During the luteal phase of the estrous cycle as well as in early pregnancy, there is a large increase in the amount of protein secreted by the uterus. Knight et al. (1973) examined the effects of progesterone and/or estradiol-17B on recoverable protein from uterine flushings of ovariectomized gilts. A progesterone dose of 2.2mg/Kg body weight for 15 days resulted in a five-fold increase in the amount of total recoverable protein when compared to corn oil treated controls or estrogen treated gilts. A fourteen-fold increase was observed when 1.1μg of estradiol-17B was administered in concert with the previously mentioned progesterone dosage. The synergistic effect of pregesterone and estrogen on uterine secretions has also been reported in the rat (Surani, 1977) and mouse (Aitken, 1977; Fishel, 1979).

Murray et al. (1972) and Squire et al. (1972) demonstrated both qualitative and quantitative changes in uterine proteins from nonpregnant gilts during the estrous cycle. The major quantitative change in total recoverable protein, i.e., the amount of protein recovered from a single flush of each uterine horn, occurred between days 12 and 15 of the cycle. This period corresponds to the late luteal phase of the cycle when peripheral plasma progesterone levels reach maximal levels. Sephadex G-200 gel filtration was utilized to examine qualitative changes (Murray et al., 1972). Three

protein fractions (Fractions I-III) were present during the entire estrous cycle and had molecular weights greater than 200,000, 200,000 and 90,000, respectively. Between days 9 and 16 of the cycle, two additional protein fractions (Fractions IV and V) were observed. Fraction IV appeared between days 12 and 16 and imparted a curious purple opalescence to the uterine flushings. The molecular weight of this protein was estimated to be 45,000 while Fraction V was observed as early as day 9 and had an estimated molecular weight of 20,000. Polyacrylamide gel electrophoresis revealed similar findings (Squire et al., 1972). Bazer (1975) reiterated the implications of these findings by pointing out that the changes in uterine luminal proteins are related at least temporally to three major physiological occurrences: 1) these changes take place during the luteal phase of the estrous cycle when progesterone levels are maximal (Hansel and Echternkamp, 1972); 2) in the pregnant animal, these alterations occur during the period of rapid blastocyst elongation (Perry and Rowlands, 1962); and 3) in the absence of pregnancy, luteal regression begins on day 15. In an effort to elucidate the role of these progesterone-induced proteins more elaborate studies were initiated.

Purification and physical properties

The protein initially identified as Fraction IV and collected from gilts between days 12 and 16 of the cycle (Murray et al., 1972; Squire et al., 1972) was purified by

Chen et al. (1973). Uterine flushings were obtained from both normal gilts as well as ovariectomized progesterone/estrogen treated gilts. The protein was purified by successive chromatography on CM-cellulose and Sephadex G-100. After elution from the Sephadex G-100, the protein exhibited a single band which ran toward the cathode during electrophoresis on polyacrylamide gels. Molecular weight of the purified protein was estimated by both gel electrophoresis and equilibrium centrifugation with the average value calculated to be 32,500. The carbohydrate content of the purple protein was estimated to be 12.5% by weight. Further analysis of this protein by Schlosnagle et al. (1974) revealed that it contains iron (1 iron atom/32,000 molecular weight) and exhibits acid phosphatase activity.

More recent studies by Brumbaugh et al. (1979) and Buhi et al. (1979) indicate a slightly higher molecular weight estimate of approximately 35,000. These values are lower than estimates by Campbell et al. (1978) and Keough et al. (1980) who suggested a molecular weight of 40,000. In addition, they concluded that the purple phosphatase contained two iron atoms per molecule in contrast to earlier reports of one. Due to these conflicting data, Buhi et al. (1979) reexamined the purple phosphatase, which is now referred to as uteroferrin, by a colorimetric procedure as well as atomic absorption spectrometry. Both methods indicated a molecular weight of 32,000 to 35,000 based on dry weight of protein, which is consistent with one iron atom rather than two per molecule.

The acid phosphatase activity of the early pregnant uterus increased rapidly as noted by Zavy (1979). Schlosnagle et al. (1974) tested the phosphatase activity of uteroferrin by measruing the hydrolysis of p-nitrophenylphosphate. Activity was found to be approximately 35 to 40 µmol nitrophenol released/min/mg protein with the reaction rate linear for up to 10 minutes. The pH optimum was found to be 4.9 using 0.1M acetate buffer and the Km was determined to be 2.2 ± 0.3 mM. In the presence of such activators as B-mercaptoethanol and ascorbate, the acid phosphatase activity of uteroferrin was greatly enhanced. However, the activity of this enzyme to naturally occurring substrates such as glucose-6-phosphate and glycerol phosphate is minimal (Schlosnagle et al., 1974; Roberts et al., 1976a).

Uteroferrin has an extinction maximum near 545 nm (Schlosnagle et al., 1974). Addition of B-mercaptoethanol results in a shift to 510 nm, producing a pink form of the protein, with a concomitant increase in enzymatic activity. In both the purple and the pink forms, iron was in the ferric (Fe^{+3}) form. The activation was attributed to a confirmational change around the iron binding site (Schlosnagle et al., 1976). Prolonged treatment with mercaptoethanol resulted in loss of color and enzymatic activity as well as release of free ferrous ions. Color and phosphatase activity could be restored by addition of Fe^{+3} while Fe^{+2} was ineffective. This remains a point of controversy, however, since Keough et al. (1980) indicated that Fe^{+2} was more effective

in restoration of enzymatic activity. When reconstitution of the apo-form of uteroferrin was attempted with high concentrations of Fe^{+3}, there was no restoration of acid phosphatase activity. When only a small excess of Fe^{+3} was utilized, however, a significant return of activity was observed. It is possible that at higher concentrations of Fe^{+3} utilized, the enzyme system may have been poisoned by the excess of metal ion. The same authors indicated that the addition of Zn^{+2} restored a major portion of enzymatic activity while Cu^{+2} was ineffective. This is contradictory to earlier work (Schlosnagle et al., 1976) which demonstrated the partial effectiveness of Cu^{+2}. It is obvious that further studies are necessary in this area.

Synthesis and distribution of uteroferrin

Chen et al. (1973) prepared antibody to uteroferrin in sheep and utilized it to demonstrate that uteroferrin is indeed uterine specific. The purified protein as well as the crude uterine flush from progesterone treated animals yielded a single precipitation line with sheep antiserum in Ouchterlony double diffusion analysis. The antiserum did not cross-react with extracts of homogenized oviduct, heart, intestine, lung, liver, spleen or kidney tissue from gilts. Subsequent studies (Chen et al., 1975), utilizing fluorescein-labeled specific antibody to uteroferrin demonstrated that epithelial cells of the endometrial surface and uterine glands were the sites of synthesis of uteroferrin in both

pregnant and nonpregnant gilts. In the pregnant animal, placental areolae were highly fluorescent as well as cells of the chorioallantois of mesodermal origin.

These data are in direct support of earlier work by Brambell (1933) who postulated that the areolae are specialized structures involved in absorption of uterine secretions. During the course of development, the epitheliochorial placenta of the pig is forced into apposition with the maternal endometrium. This occurs between days 20 and 30 of gestation due to the rapid accumulation of allantoic fluid (Knight et al., 1974). The placental areolae form in apposition to the openings of uterine glands in the endometrium. Earlier studies by Palludan et al. (1969) using autoradiography and Wislocki and Dempsey (1946) using histochemical techniques also demonstrated the localization of iron in these areas of the porcine placenta.

Uteroferrin begins to accumulate in allantoic fluid of pregnant gilts after day 30 of pregnancy (Bazer et al., 1975). This protein was absent from the allantois prior to this time, i.e., before formation of areolae, confirming the role of the areolae in placental exchange of nutrients. As measured by acid phosphatase activity, uteroferrin reached maximal levels in allantoic fluid on our about day 60 of gestation and then declined toward term (Bazer et al., 1975). Uteroferrin can also be isolated from uterine flushings of pseudopregnant gilts. When estradiol-17B is administered on days 11 through 15 of the estrous cycle (day of

onset of estrus = day 0), corpora lutea are maintained and progesterone production continues and allows for continuation of uterine secretory activity (Frank et al., 1978). The estradiol is believed to simulate increased estrogen synthesis by the blastocyst which is the reputed signal for the maternal recognition of pregnancy (Perry et al., 1976; Bazer and Thatcher, 1977). Basha et al. (1980) indicated that the amount of uteroferrin recovered from gilts on day 60 of pseudopregnancy is comparable to that recovered from ovariectomized, progesterone treated animals; over 250 mg of purified uteroferrin (Schlosnagle et al., 1974). Basha et al. (1979) demonstrated the synthesis and secretion of uteroferrin in vitro. Organ explants cultured from pregnant gilt endometrium were utilized. It was estimated that during midgestation the endometrium has the capacity to produce a minimum of 1g of uteroferrin per day.

In summary, uteroferrin is produced by the maternal endometrium, moves across the placenta at specialized areas known as areolae and is sequestered in the allantoic fluid. Buhi et al. (1979) indicated that when uteroferrin is incubated in vitro in day 60 allantoic fluid, it has a half life of 2 to 4 days. Once the protein releases its iron, it is rapidly degraded, most likely by proteases. Allantoic fluid appears, therefore, to provide the necessary environment for the release of uteroferrin-bound iron. Upon release, the iron then appears to be bound by a transferrin like molecule (W.C. Buhi, personal communication). Since the allantois

arises as a diverticulum of the fetal hind gut and is lined with gut epithelium (Bodmer, 1968), it may behave in a manner similar to the intestinal epithelium in iron absorption. Lecce (1966) has demonstrated that the neonatal gut epithelium is capable of macromolecular absorption. Protein bound iron accumulated in the allantoic fluid may be absorbed by the allantoic epithelium and thus made available for uptake and utilization by the developing porcine conceptus.

Of the several roles postulated for uteroferrin (Schlosnagle et al., 1974; Bazer et al., 1975; Roberts and Bazer, 1980), that of an iron transport protein seems most likely. The major role of uteroferrin does not appear to be that of an acid phosphatase due to its low enzymatic activity at physiological pH and moderately high Km.

In addition to large amounts of uteroferrin produced by the uterus, its accumulation in the allantoic fluid and high turnover rate, Ducsay (1977) indicated that iron levels in the allantoic fluid appear to follow a similar pattern to the levels of uteroferrin. In the same study it was also determined that iron treatment of the dam during the periods of peak production of uteroferrin lead to increases in fetal as well as neonatal iron stores. Thus it appears that uteroferrin may play a major role in maternal to fetal iron transport in swine.

CHAPTER III
CHARACTERIZATION OF FETAL IRON DEPOSITION AND UTEROFERRIN CONTENT DURING GESTATION

Introduction

Ducsay (1977) outlined iron deposition in porcine fetal tissues and allantoic fluid between days 30 and 90 of gestation. In that study, significant increases in iron content were demonstrated in fetal liver as well as the fetal carcass minus the liver. It was suggested that a temporal relationship existed between levels of iron and uteroferrin in allantoic fluid, with the implication that uteroferrin may play a role in iron transport. However, no actual measurement of uteroferrin in allantoic fluid was made. In addition, neither placental nor amniotic fluid iron content was measured. Lopez (1975) examined changes in amniotic and allantoic fluid iron, but levels were not related directly to fetal iron stores or uteroferrin levels.

This study was designed to examine the relationship between iron and uteroferrin levels in allantoic fluid. In addition, changes in placental iron and uteroferrin levels as well as fetal liver iron were examined during gestation. By measuring levels of iron and uteroferrin during the course of gestation, it might be possible to aid in elucidation of the role uteroferrin may play in iron transport in pregnant swine.

Materials and Methods

Experimental Design

Twenty-one sexually mature crossbred gilts of similar age, weight and genetic background were checked twice daily for estrous behavior using mature boars. Gilts were bred at 12 and 24 hours after onset of estrus and randomly assigned to hysterectomy on either day 30, 45, 60, 75, 90, 105 or 112 of gestation for collection of fetal tissues and fluids.

Procedure

Surgery was performed on the respective days with anesthesia induced by sodium thiopental and maintained with methoxyflurane (Pittman-Moore). The reproductive tract was exposed by midventral laparotomy, and the intact uterus, ovaries and a portion of the cervix were removed. After hysterectomy, the uterus was opened along the mesometrial border, and each fetal-placental unit was removed intact.

The chorioallantois was punctured with an 18 gauge needle, and fluid free of contamination was collected in a 20 ml syringe. The remaining allantoic fluid from each fetus was collected to measure total allantoic fluid volume. A similar procedure was utilized for collection of amniotic fluid. However, no samples of AMF were collected on day 30 due to lack of fluid on this day of gestation. Fluid samples were stored at -20 C until analyzed.

Each fetus was freed of surrounding membranes, and the liver was removed. The fetal liver, fetus minus liver and placentae were blotted to remove excess blood. Tissues were weighed and then stored at -20 C for future analysis. In addition, samples of endometrium were stripped from the underlying myometrium, and both tissues were weighed to estimate the fraction of uterine weight comprised of endometrium. Endometrium was also stored at -20 C until analysis.

Sample Preparation

Glassware. Porcelain crucibles were soaked for approximately 24 hours in dichromate-sulfuric acid solution, rinsed three times in tap water, soaked in 10% HCl and rinsed again three times in deionized water. All funnels and volumetric flasks were soaked overnight in detergent solution and rinsed with tap water followed by three rinses with deionized water. Glassware was subsequently soaked in 10% HCl overnight and then rinsed three times with deionized water.

Tissue. Tissue samples were processed according to methods outlined by Ducsay (1977). Each fetal liver was thawed to room temperature, placed into a clean, preweighed crucible and dried in an oven at 100 C for a minimum of 16 hours. The crucibles were allowed to cool for 2 hours in a dessicator, then weighed to a constant weight.

Crucibles containing dry tissue samples were placed on a hot plate (200 C) under a fume hood and pre-ashed with the addition of 50% HNO_3. Pre-ashed samples were then placed in a muffle furnace at 250 C. The temperature was increased 100 C/hour until a final temperature of 550 C was attained. Samples remained at 550 C for 18 hours. After cooling the ashed samples were weighed.

Crucibles containing the ash residue were placed on a hot plate (200 C) for acid hydrolysis. The ash was moistened with a few drops of deionized water, and approximately one-third volume of each crucible was filled with 50% HNO_3. After partial evaporation, 10% HNO_3 was added followed by deionized water. The ash solution was quantitatively transferred through sharkskin filter paper into volumetric flasks, mixed by inversion and stored in Falcon tubes.

Each fetus minus liver and placenta was homogenized in a Waring blender until a homogeneous mixture was achieved. A sample of approximately 30 g of each homogenate was placed in a crucible and treated as previously described for the fetal liver samples. Endometrial samples were also handled like the fetal livers.

Fetal fluids. Aliquots of 5 ml of allantoic and amniotic fluid were pipetted into clean porcelain crucibles and oven dried at 100 C. The dried fluid samples were ashed in a muffle furnace, then treated in the same manner as the tissue samples.

Iron Analyses

Iron concentration for all samples was measured by atomic absorption spectrophotometry (Perkin-Elmer Model 306). Standard operating conditions described by the manufacturer were utilized. Blanks and standard reference solutions were used to calculate standard curves. Iron concentrations of the samples were calculated by interpolation from standard curves by linear regression.

Acid Phosphatase Assays

Acid phosphatase activity of allantoic fluid was measured according to the procedure described in Sigma Technical Bulletin No. 85 (1971) (Appendix A).

Placental Extracts

Approximately 10 g of placental homogenate from each placenta were further homogenized in a Polytron (Brinkman) with one-half the tissue weight of deionized water added. After homogenization, samples were centrifuged in a refrigerated centrifuge at 15,000 rpm for 20 minutes. The volume of the supernatant was measured to calculate g placental tissue/ml extract. Samples were stored at -20 C until analyzed.

Hemoglobin Determinations

Fetal hemoglobin levels were measured by the cyanomethemoglobin reagent method (Crosby et al., 1954). A

20µl blood sample was pipetted in 4.98ml of cyanomethemoglobin reagent (Hycel), and optical density was determined at 570 nm. Hemoglobin levels (g/100ml blood) were calculated from a standard curve constructed utilizing a cyanomethemoglobin standard.

Uteroferrin Radioimmunoassay

The procedure utilized for quantitative determination of uteroferrin was described by Buhi (1980). A double antibody procedure was used. The primary antibody was raised in sheep by sequential intradermal injections of uteroferrin and Freund's adjuvant (Chen et al., 1975). The secondary antibody was prepared by sequential injections of sheep γG and Freund's adjuvant into male rabbits. No cross reactivity of the primary antibody was observed when tested against porcine transferrin or porcine lactoferrin by Ouchterlony immunodiffusion analysis. Radiolabeled uteferrin was prepared by incubating purified uteroferrin with ^{125}I in the presence of Iodogen (Pierce) (Markwell and Fox, 1978). The labeled protein was separated from free ^{125}I by successive column chromatography on Sephadex G-50 and G-100 (Pharmacia) columns. Standard curves were prepared from stock samples of purified uteroferrin (100µg/ml) by serial dilution with assay buffer. Samples were diluted with assay buffer in the range of the standard curve and were run in duplicate. Sensitivity of the assay ranged

from .25 to .50μg/ml. Details of the assay procedure appear in Appendix A. Validation is incomplete at this time.

Statistical Analysis

Data were analyzed by method of least squares analysis of variance utilizing Statistical Analysis System (Barr et al., 1979). Changes in various fetal parameters were examined over days of gestation relative to variation among gilts within day of gestation. Expected mean squares in analysis of variance of the data are listed in Table 3.1.

TABLE 3.1. ANALYSIS OF VARIANCE
EXPECTED MEAN SQUARES

Source	df	Expected mean square
Day	6	$\sigma^2_e + K_2\sigma^2_{G(D)} + K_3\sigma^2_D$
Gilt (Day)	14	$\sigma^2_e + K_1\sigma^2_{G(D)}$
Residual	N-21	σ^2_e

The number of observations (N) may vary due to insufficient sample size or loss of samples during analysis. Polynomial regression equations for the various fetal parameters measured across day of gestation appear in Appendix B.

Results and Discussion

Weight of Fetal Tissues

Liver and fetus minus liver. Weight of fetal liver and fetus minus liver increased ($P<.01$) over the days of gestation examined (Table 3.2). These data are consistent with earlier findings of Ullrey et al. (1965) and Robinson (1976) who found fetal liver as well as the entire fetus to exhibit curvilinear growth to approximately day 70 of gestation followed by growth in a linear manner.

Placenta. Placental weight was found to be significantly ($P<.01$) affected by day of gestation (Table 3.2). The major increase in placental weight occurred between days 30 and 60 and then remained relatively constant to day 112 of gestation. These data are in agreement with earlier studies (Ducsay, 1977; Knight et al., 1977). Fetal growth depends on the degree of placental development. Knight et al. (1977) found that gilts with experimentally reduced placental surface area demonstrated a decrease in fetal growth. The relationship of the placenta to fetal nutrition, specifically iron transport, will be discussed further in this chapter.

Tissue Iron

Liver. Iron concentration and total iron content of fetal livers during gestation are depicted in Figure 3.1. Iron concentration was affected by day of gestation ($P<.05$).

TABLE 3.2. CHANGES IN WEIGHT OF THE FETAL LIVER, FETUS MINUS LIVER AND PLACENTA AT VARIOUS STAGES OF GESTATION[a]

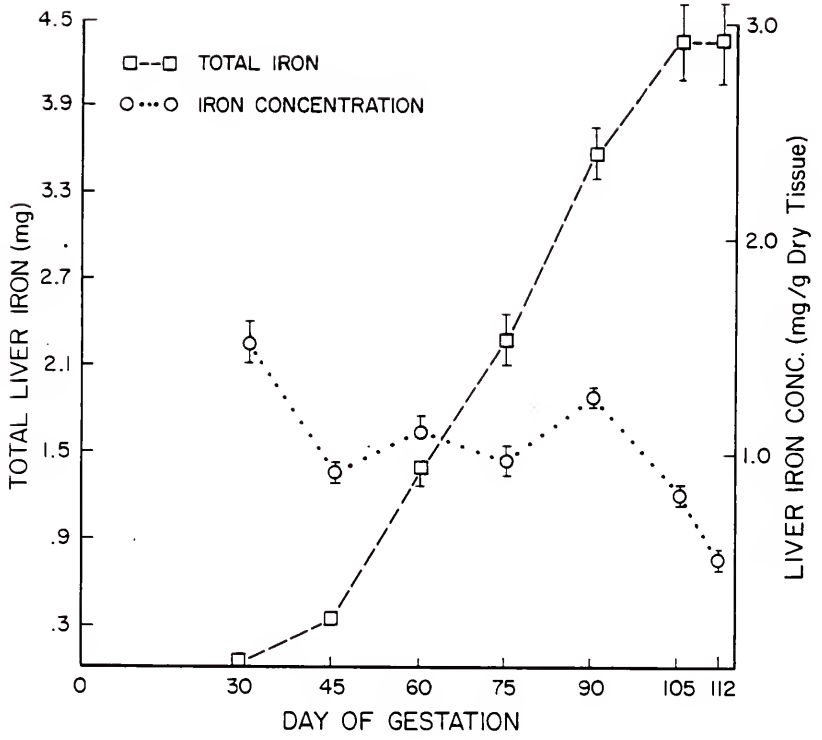
Day of gestation	N[b]	Liver weight[c] (b)	Fetus minus liver[c] (g)	Placenta[c] (g)
30	36	.22 ± .01	1.33 ± .03	25.34 ± 1.32
45	28	2.35 ± .12	19.64 ± .57	80.46 ± 6.02
60	28	7.39 ± .19	118.45 ± 3.83	233.69 ± 17.11
75	28	13.19 ± .57	309.39 ± 9.76	225.94 ± 16.18
90	24	19.69 ± .87	596.15 ± 16.62	208.97 ± 10.45
105	27	27.18 ± 1.0	861.57 ± 30.85	237.04 ± 13.63
112	30	40.29 ± 1.41	1086.88 ± 42.42	237.45 ± 8.22

[a]All values represent means ± standard errors.

[b]N = number of observations.

[c]Least squares analysis of variance indicated significant ($P < .01$) day effects.

Figure 3.1. Total iron content (mg) and iron concentration (mg/g dry tissue) of fetal livers during gestation. Both parameters were affected by day of gestation ($P < .01$ and $P < .05$, respectively).


TOTAL IRON
IRON CONCENTRATION
TOTAL LIVER IRON (mg)
LIVER IRON CONC. (mg/g Dry Tissue)
4.5
3.9
3.3
2.7
2.1
1.5
.9
.3
3.0
2.0
1.0
0
30
45
60
75
90
105
112
DAY OF GESTATION

A decline in concentration was observed between days 30 and 45 of gestation. This decrease occurs when fetal hematopoesis is very active (Wadill et al., 1962; Hoskins and Hansard, 1964a). Between days 45 and 90 of gestation, iron concentration remains relatively static, followed by a decline on days 105 and 112 of gestation. This decline is in marked contrast to total fetal liver iron (Figure 3.1). Total iron content of the fetal liver increased between days 30 and 112 of gestation ($P<.01$). The greatest increase in total iron content was observed between days 45 and 90. Since iron concentration remained relatively constant during this time, the increase in total iron content can be attributed to changes in weight of the fetal liver (Table 3.2) and a relatively constant rate of iron deposition per unit increase in liver weight.

Fetus minus liver. Iron concentration and total iron content of the fetus minus liver are listed in Table 3.3. Iron concentration was affected by day of gestation ($P<.05$) and followed a pattern of change similar to that observed for the fetal liver. Total iron content increased over gestation ($P<.01$). Analogous to the liver, the change in total iron content of the fetus minus liver can be attributed to an increase in fetal weight and maintenance of a fairly constant rate of tissue iron deposition as previously described by Robinson (1976).

Placenta. Placental iron concentration as well as total placental iron changed significantly ($P<.05$ and $P<.01$,

TABLE 3.3. CHANGES IN IRON CONTENT OF THE FETUS MINUS LIVER AT VARIOUS STAGES OF GESTATION[a]

Day of gestation	N[b]	Fetus minus liver	
		Iron concentraction[c] (µg/g dry tissue)	Total iron content[d] (mg)
30	33	474.76 ± 46.33	.04 ± .01
45	26	254.63 ± 16.20	.44 ± .03
60	28	153.53 ± 16.28	1.79 ± .19
75	27	115.08 ± 5.16	3.73 ± .20
90	17	200.77 ± 7.63	14.67 ± .71
105	23	112.97 ± 5.71	14.84 ± .54
112	18	119.20 ± 7.74	23.53 ± 1.75

[a]All values represent means ± standard errors.

[b]N = number of observations.

[c]Least squares analysis of variance indicated significant ($P < .05$) day effects.

[d]Least squares analysis of variance indicated significant ($P < .05$) day effects.

respectively) as gestation progressed (Table 3.4). Both concentration and total iron content increased to day 105 of gestation with a subsequent decline on day 112. Hansard (1965) measured total ^{59}Fe deposited in the placenta from the maternal circulation and found that iron levels increased steadily to day 105 of gestation. Later studies by Palludan et al. (1969) revealed a similar finding, suggesting that the placenta plays a role not only in iron transport, but iron storage as well.

Fetal hemoglobin. Fetal hemoglobin levels increased ($P<.05$) during gestation (Table 3.5). Due to an insufficient volume of blood on day 30, no hemoglobin determinations were made until day 45. As previously described, fetal hematopoesis is quite active between days 45 and 60 as evidenced by the rapid increase in hemoglobin levels during this period of gestation. Hemoglobin levels continued to increase until day 90. Beyond this point, levels remained relatively constant.

Endometrium. The percent of total weight of empty uterus comprised of endometrium is listed in Table 3.6. Total estimated endometrial weight is also listed. Both parameters were significantly ($P<.01$) affected by day of gestation. Endometrial iron concentration changed ($P<.05$) over days of gestation while total endometrial iron content remained relatively constant (Table 3.6). It is of interest that the endometrium comprises the largest percentage of empty uterine weight on day 60 of gestation. This is the

TABLE 3.4. CHANGES IN PLACENTAL IRON CONTENT AT VARIOUS STAGES OF GESTATION[a]

Day of gestation	N[b]	Placenta	
		Iron concentration[c] (μg/g dry tissue)	Total iron[d] (mg)
30	25	300.05 ± 17.17	.26 ± .02
45	24	338.85 ± 12.03	1.77 ± .15
60	24	395.64 ± 21.54	4.56 ± .33
75	26	407.98 ± 23.06	6.76 ± .67
90	21	413.19 ± 16.23	6.06 ± .38
105	27	485.39 ± 30.42	9.09 ± .81
112	30	288.14 ± 10.48	5.07 ± .28

[a]All values represent means ± standard errors.

[b]N = number of observations.

[c]Least squares analysis of variance indicates significant ($P < .05$) day effects.

[d]Least squares analysis of variance indicates significant ($P < .01$) day effects.

TABLE 3.5. CHANGES IN FETAL HEMOGLOBIN LEVELS AT VARIOUS STAGES OF GESTATION[a]

Day of gestation	N[b]	Fetal hemoglobin[c] (b/100ml blood)
30	---	---
45	15	6.80 ± .45
60	27	8.04 ± .31
75	29	8.89 ± .22
90	13	9.71 ± .59
105	27	9.53 ± .31
112	30	9.75 ± .23

[a]All values represent means ± standard errors.

[b]N = number of observations.

[c]Least sequares analysis of variance indicate significant ($P < .05$) day effects.

TABLE 3.6. CHANGES IN ENDOMETRIAL WEIGHT AND IRON CONTENT AT VARIOUS STAGES OF GESTATION[a]

Day of gestation	N	Endometrium			
		% endometrium[c]	Total endometrial weight (g)	Iron concentration[d] (mg/g)	Total iron (mg/uterus)
30	3	40.38 ± .81	543.50 ± 58.84	.68 ± .03	372.70 ± 62.09
45	3	51.35 ± 2.05	711.40 ± 34.90	.34 ± .08	317.75 ± 75.75
60	3	51.16 ± 4.21	611.83 ± 94.23	.49 ± .09	368.50 ± 37.02
75	3	48.25 ± 1.58	839.33 ± 220.45	.55 ± .05	548.90 ± 197.81
90	3	45.00 ± 3.42	929.77 ± 159.83	.43 ± .04	550.90 ± 180.00
105	3	44.69 ± 3.26	1386.53 ± 142.53	.58 ± .10	663.13 ± 207.65
112	3	32.79 ± 1.62	1306.37 ± 51.62	.69 ± .11	566.95 ± 430.5

[a]All values represent means ± standard errors.

[b]N = number of observations

[c]Least squares analysis of variance indicated significant ($P < .01$) day effects.

[d]Least squares analysis of variance indicated significant ($P < .05$) day effects.

period of time when endometrial production of uteroferrin is maximal (Basha et al., 1979). The ratio of progesterone to estrogen (Knight et al., 1977) was also highest during this period of gestation. The increase in relative amounts of endometrial tissue may be due to effects of progesterone on glandular proliferation of endometrial epithelium.

Fetal Fluid Volume and Iron Content

Amniotic fluid. Volume of amniotic fluid was affected ($P<.01$) by day of gestation. Amniotic fluid volume, first measured on day 45, increased to a peak on day 75 and then declined to day 112 to volumes comparable to those observed on day 45 of gestation (Table 3.7). These data are similar to those observed by Knight et al. (1977). It is interesting to note the large decline in amniotic fluid volume as the fetuses approached term. During this period of gestation, the major increase in weight of the fetus is due to increased moisture content (Robinson, 1976). This fluid pool may supply the fetus with additional water during this phase of growth.

Iron concentration in amniotic fluid did not change during gestation, while total iron content was affected ($P<.05$) by day of gestation (Table 3.7). Therefore, changes in total amniotic fluid iron are due to alterations in amniotic fluid volume and not changes in iron concentration. The sharp decline in iron content of amniotic fluid in late gestation might be attributable to swallowing by the

TABLE 3.7. CHANGES IN VOLUME AND IRON CONTENT OF AMNIOTIC FLUID DURING VARIOUS STAGES OF GESTATION[a]

Day of gestation	N[b]	Amniotic fluid		
		Volume[c] (ml)	Iron concentration (µg/ml)	Total iron[d] (µg/amniotic sac)
30		---	---	---
45	28	25.79 ± .77	4.74 ± .86	121.04 ± 22.69
60	28	132.61 ± 7.39	2.79 ± .33	375.35 ± 55.71
75	26	196.35 ± 12.51	4.82 ± 1.01	1068.95 ± 290.46
90	24	171.70 ± 12.78	5.64 ± 1.09	918.54 ± 153.98
105	22	106.15 ± 16.40	3.48 ± .04	382.07 ± 76.06
112	27	57.41 ± 7.81	3.87 ± .22	231.39 ± 40.72

[a]All values represent means ± standard errors.

[b]N = number of observations.

[c]Least squares analysis of variance indicated significant ($P < .01$) day effects.

[d]Least squares analysis of variance indicated significant ($P < .05$) day effects.

fetus. Mistretta and Bradley (1975) indicated that this would alter composition as well as volume of this fluid pool.

Allantoic fluid. Allantoic fluid volume changed significantly ($P<.01$) during gestation (Table 3.8). These findings are similar to earlier studies by Knight et al. (1977) which indicated a rapid accumulation of allantoic fluid prior to day 30 of gestation at which time an initial fluid peak is reached. This is followed by a decline in volume by day 45 with a subsequent increase to another peak on day 60. Beyond this stage of gestation allantoic fluid levels were observed to decline to day 112. These fluctuations in fluid volume have been associated with alterations in permeability of the placenta and changes in sodium: potassium ratios (Goldstein, 1977). The ratio was greater than one during periods of allantoic fluid accumulation (days 20-30 and 55-65) and less than one when low fluid volumes were observed (days 35-50 and 70-100). These data suggested that placental water movement, i.e., allantoic fluid accumulation, is associated with ion movement.

Iron concentration and total iron content of allantoic fluid exhibited a similar pattern of change during gestation (Table 3.8). Both parameters were affected ($P<.01$) by day of gestation. Iron concentration reached a peak level of 4.63µg/ml on day 75 of gestation while total allantoic fluid iron levels were highest on day 60. It is of interest to note that this is the same period of

TABLE 3.8. CHANGES IN VOLUME AND IRON CONTENT OF ALLANTOIC FLUID AT VARIOUS STAGES OF GESTATION[a]

Day of gestation	N[b]	Allantoic fluid		
		Volume[c] (ml)	Iron concentration[c] (µg/ml)	Total iron[c] (µg/allantoic sac)
30	35	197.63 ± 8.68	1.56 ± .07	307.25 ± 23.01
45	26	127.65 ± 25.19	4.10 ± .67	395.17 ± 71.98
60	26	304.12 ± 40.30	4.25 ± .41	1247.35 ± 208.27
75	28	119.89 ± 23.15	4.63 ± .42	469.71 ± 73.18
90	24	66.17 ± 19.10	4.38 ± .36	206.84 ± 31.15
105	27	47.48 ± 12.97	2.53 ± .52	154.15 ± 48.68
112	27	30.96 ± 7.67	3.85 ± .68	193.34 ± 68.96

[a]All values represent means ± standard errors.

[b]N = number of observations.

[c]Least squares analysis of variance indicated significant ($P < .01$) day effects.

gestation during which uteroferrin levels are highest in allantoic fluid (Bazer et al., 1975). Ducsay (1977) suggested that the presence of uteroferrin in allantoic fluid might explain observed fluctuations in iron content of this fluid pool. It was further suggested that iron present in allantoic fluid may be available for uptake and utilization by the developing conceptus. However, uteroferrin levels were not examined.

Acid Phosphatase

Allantoic fluid. Concentration as well as total allantoic fluid acid phosphatase were affected ($P<.01$) by day of gestation (Table 3.9). Total acid phosphatase activity was highest on day 60 of gestation. These data are consistent with earlier work (Chen et al., 1975; Bazer et al., 1975). Over 94% of the acid phosphatase activity in allantoic fluid can be attributed to uteroferrin (Schlosnagle et al., 1974).

Figure 3.2 demonstrates the relationship between total iron content and acid phosphatase activity (as a measure of uteroferrin content) in allantoic fluid during gestation. Both parameters follow the same pattern of accumulation and depletion, with peak levels occurring on day 60 of gestation. This implies a relationship between uteroferrin and iron content of allantoic fluid. It appears that iron is carried across the placenta by uteroferrin and is sequestered in allantoic fluid for possible uptake and utilization by the developing conceptus.

TABLE 3.9. CHANGES IN ALLANTOIC FLUID ACID PHOSPHATASE LEVELS AT VARIOUS STAGES OF GESTATION[a]

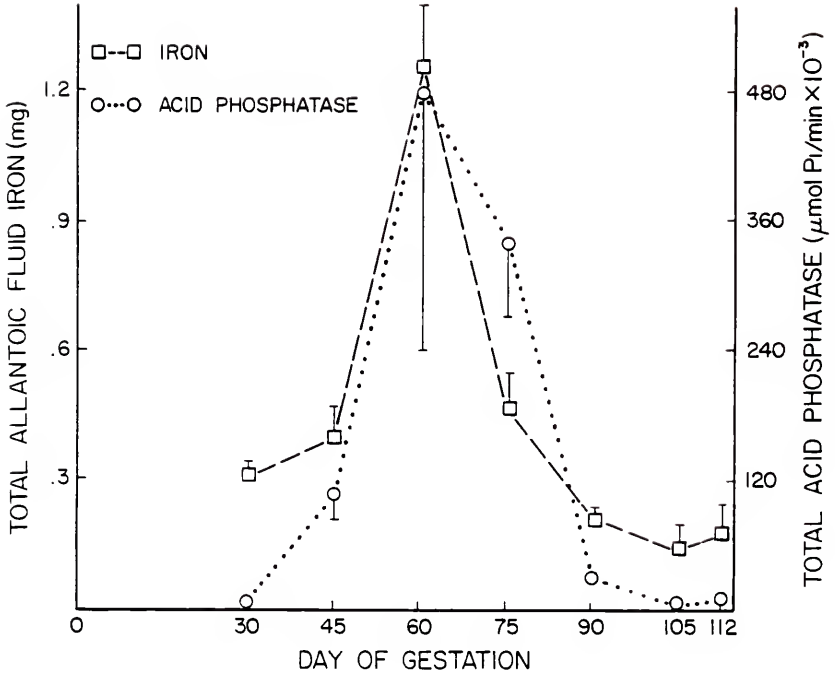
Day of gestation	N[b]	Allantoic fluid	
		Acid phosphatase concentration[c] (μmol Pi/min/ml)	Total acid phosphatase (μmol Pi/min/allantoic fluid sac x 10^{-3})
30	32	2.00 ± 3.91	.30 ± .10
45	25	1526.20 ± 309.88	108.63 ± 27.85
60	28	1135.27 ± 177.70	488.51 ± 241.36
75	28	4886.58 ± 1275.73	355.53 ± 84.92
90	24	1507.68 ± 621.57	33.82 ± 12.41
105	19	24.91 ± 10.81	1.26 ± .76
112	23	231.75 ± 59.21	9.49 ± 2.61

[a]All values represent means ± standard errors.

[b]N = number of observations.

[c]Least squares analysis of variance indicated significant ($P < .01$) day effects.

Figure 3.2. Total iron content (mg) and acid phosphatase activity (µmol Pi/min/allantoic sac x 10^{-3}) in allantoic fluid during gestation.


IRON
ACID PHOSPHATASE
TOTAL ALLANTOIC FLUID IRON (mg)
1.2
.9
.6
.3
0
480
360
240
120
TOTAL ACID PHOSPHATASE (μmol Pi/min ×10⁻³)
30
45
60
75
90
105
112
DAY OF GESTATION

Placenta. Placental acid phosphatase activity during gestation is summarized in Table 3.10. Acid phosphatase concentration (μmol Pi/min/g tissue) changed over gestation ($P<.05$), while total placental acid phosphatase was not affected by day. The relationship between total placental acid phosphatase and total placental iron is illustrated in Figure 3.3. The pattern of iron accumulation in the placenta follows a pattern similar to that observed for placental acid phosphatase activity during gestation. Analogous to patterns observed in allantoic fluid (Figure 3.2), the association between iron and acid phosphatase levels in the placenta implies a relationship between iron and uteroferrin. The placenta may store uteroferrin since levels remain relatively constant. However, unlike the relationship between acid phosphatase and uteroferrin established in allantoic fluid (Schlosnagle et al., 1974), the amount of placental acid phosphatase attributable to uteroferrin is unknown. To circumvent this problem, a specific radioimmunoassay for uteroferrin was utilized.

Placental Uteroferrin

Uteroferrin levels in placental tissue extracts measured by radioimmunoassay are listed in Table 3.11. Both concentration and total uteroferrin content of placenta changed during gestation ($P<.01$). Large increases in placental uteroferrin levels were observed between days 45 and 75, after which time, levels remained relatively

TABLE 3.10. CHANGES IN PLACENTAL ACID PHOSPHATASE LEVELS AT VARIOUS STAGES OF GESTATION[a]

Day of gestation	N[b]	Placenta	
		Acid phosphatase concentration[c] (μmol Pi/min/g tissue)	Total acid phosphatase (μmol Pi/min/placenta x 10^{-3})
30	--	---	---
45	27	181.53 ± 35.31	11.39 ± 1.48
60	26	60.62 ± 10.11	14.37 ± 3.12
75	26	70.09 ± 7.58	15.42 ± 1.46
90	17	41.71 ± 4.85	8.08 ± 1.04
105	27	63.17 ± 4.90	15.36 ± 1.13
112	30	60.89 ± 4.06	14.14 ± .87

[a]All values represent means ± standard errors.

[b]N = number of observations.

[c]Least squares analysis of variance indicated significant ($P < .05$) day effects.

Figure 3.3. Total placental iron (mg) and acid phosphatase activity (μmol Pi/min/placenta x 10^{-3}) during gestation. Total placental iron was affected by day of gestation ($P < .01$).

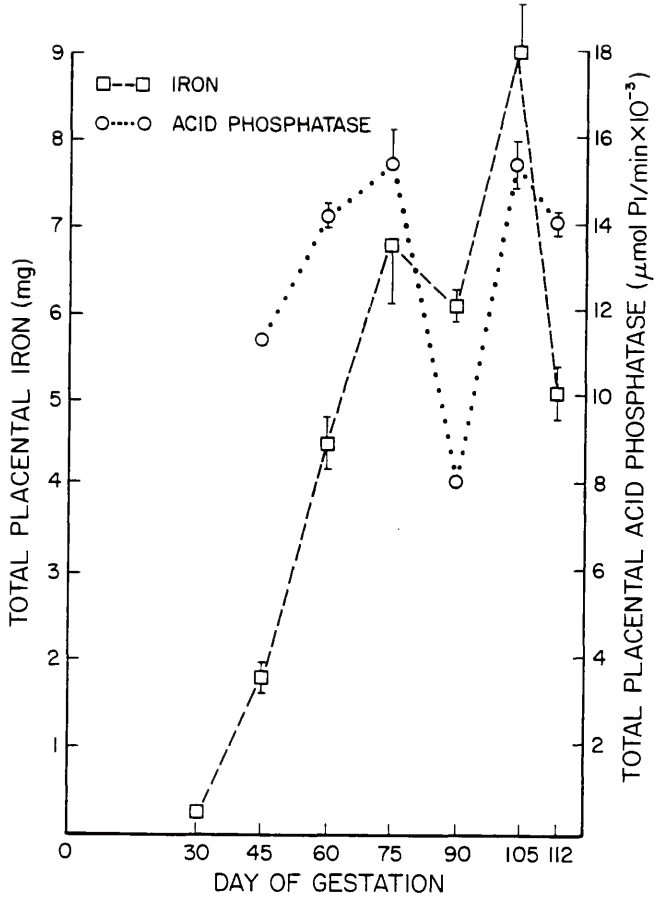

IRON
ACID PHOSPHATASE
TOTAL PLACENTAL IRON (mg)
TOTAL PLACENTAL ACID PHOSPHATASE (μmol Pi/min×10⁻³)
DAY OF GESTATION
0
30
45
60
75
90
105
112
1
2
3
4
5
6
7
8
9
2
4
6
8
10
12
14
16
18

TABLE 3.11. CHANGES IN PLACENTAL UTEROFERRIN CONTENT AT VARIOUS STAGES OF GESTATION

Day of gestation	N[b]	Placenta	
		Uteroferrin concentration[c] (mg/g tissue)	Total uteroferrin[c] (mg/placenta)
30	--	---	---
45	26	.45 ± .09	33.33 ± 2.83
60	23	.48 ± .03	113.11 ± 15.15
75	24	2.08 ± .14	492.10 ± 41.12
90	18	1.54 ± .15	309.42 ± 31.64
105	25	1.45 ± .09	386.69 ± 39.53
112	29	1.56 ± .12	376.60 ± 33.28

[a]All values represent means ± standard errors.

[b]N = number of observations.

[c]Least squares analysis of variance indicated significant P < .01) day effects.

constant. Total placental iron levels (Figure 3.3) follow a similar pattern. This fact reemphasizes the relationship between iron content and uteroferrin levels in placental tissue. Endometrial explant culture studies by Basha et al. (1979) demonstrated maximal uteroferrin production (2mg/g tissue/24 hours) from day 60 endometrial tissue. Since the endometrium from both uterine horns weighs about 600 g on day 60, a minimum of 1 g of uteroferrin can be produced daily during this period of gestation.

It appears that the placenta stores uteroferrin, since placental levels remain high during the latter part of gestation while uteroferrin production by the endometrium declines (Basha et al., 1979). As described earlier, the allantois may serve as a reservoir for excess uteroferrin bound iron which may function as a reserve for the conceptus. Based on ^{59}Fe studies, Palludan et al. (1969) postulated two iron pools in the fetal-placental unit. One pool exhibited a large turnover rate, while the other pool demonstrated a low turnover rate with iron liberated slowly, becoming available to the fetus based on its iron demands. Uteroferrin bound iron, stored in the placenta, may serve as this slow release pool. Caution must be exercised, however, since a maximum 8% of the total iron content of the placenta may be attributed to uteroferrin.

In summary, uteroferrin levels in both allantoic fluid and placental tissue follow a pattern similar to that observed for iron content. Earlier studies (Chen et al.,

1975; Bazer et al., 1975; Ducsay, 1977) postulated the possible relationship between uteroferrin and iron content of allantoic fluid. Based on this study, the placenta also appears to be involved in iron storage, specifically storage of iron transported to the placenta by uteroferrin. Validation of the uteroferrin radioimmunoassay is incomplete at this time, and due care must be taken in interpretation of these results. Placental acid phosphatase levels, although not as sensitive, still indicate high levels of uteroferrin during late gestation, in close agreement with the radioimmunoassay data.

CHAPTER IV
EFFECT OF MATERNAL IRON TREATMENT ON FETAL IRON AND UTEROFERRIN CONTENT

Introduction

Early studies by Rydberg et al. (1959) and Pond et al. (1961) indicated that injecting sows with iron-dextran during the last few weeks of pregnancy was relatively ineffective in prevention of baby pig anemia. Oral iron therapy also failed to increase the amount of iron transferred to the fetus (Miller et al., 1964).

Recent work by Ducsay (1977) revealed that iron-dextran injections much earlier in gestation (between days 40 and 60) increased the amount of iron in the fetus and neonatal hemoglobin levels. Iron concentration of allantoic fluid and total iron content of fetal livers from treated gilts were significantly ($P<.05$) higher than those from control litters on day 90 of gestation. It was suggested that providing the dam with additional iron during the period of gestation when uteroferrin levels are maximal allowed increased maternal to fetal iron transport. However, uteroferrin levels were not measured.

The aim of this study was to examine effects of iron treatment of the dam between day 40 and 60 of gestation on iron content of fetal tissues and fluids. In addition, the

relationship between uteroferrin levels and iron content of the fetal-placental unit was examined.

Materials and Methods

Experimental Design

A total of eight crossbred gilts of similar age, weight and genetic background were checked twice daily for estrous behavior using mature boars. Gilts were bred 12 and 24 hours after onset of estrus and randomly assigned to two treatment groups:

(1) Control--no supplementary iron injections during gestation.

(2) Iron Treatment--each gilt received a total of 22mg iron/kg body weight divided among five injections on days 40, 45, 50, 55 and 60 of gestation.

All gilts were hysterectomized on day 90 of gestation for collection of fetal tissues and fluids.

Procedure

Surgical procedures, tissue collection and preparation as well as various analyses were performed as described in the previous chapter. Iron was administered as iron-dextran (No-Nemic, Armour Baldwin Laboratories) by intramuscular injections into the dorsal neck region of treated gilts.

Statistical Analysis

Data were analyzed by least squares analysis of variance procedures with the Statistical Analysis System (Barr et al., 1979). Expected mean squares in the analysis of variance are listed in Table 4.1.

TABLE 4.1. ANALYSIS OF VARIANCE EXPECTED MEAN SQUARES

Source	df	Expected mean square
Treatment	1	$\sigma^2_e + K_2\sigma^2_{G(T)} + K_2\sigma^2_T$
Gilt (treatment)	6	$\sigma^2_e + K_1\sigma^2_{G(T)}$
Residual	N-8	σ^2_e

Results and Discussion

The principal aim of this study was to examine the effect of iron treatment on iron and acid phosphatase/uteroferrin in fetal liver, allantoic fluid and placenta. Additional parameters were also examined but will not be discussed since treatment effects were not observed. However, a comparison of these various measurements between treatment groups is summarized in Table 4.2.

Iron

Fetal liver. Iron concentration and total liver iron for the two treatment groups are summarized in Table 4.3.

TABLE 4.2. COMPARISION OF FETAL PARAMETERS FROM CONTROL AND TREATMENT GILTS[a]

Parameter	Treatment	
	Control	Iron treatment
Liver weight (g)	19.68 ± .86	19.01 ± .75
Fetus minus liver weight (g)	578.12 ± 15.53	548.90 ± 17.16
Placental weight (g)	196.31 ± 9.25	171.85 ± 7.05
Fetus minus liver iron concentration (µg/g dry tissue)	194.97 ± 5.45	177.43 ± 15.83
Fetus minus liver total iron (mg)	14.02 ± .62	13.37 ± 1.39
Amniotic fluid volume (ml)	154.94 ± 10.53	131.52 ± 9.44
Amniotic fluid iron concentration (µg/ml)	5.64 ± 1.09	6.25 ± 1.10
Total amniotic fluid iron (mg)	.91 ± .15	.87 ± .23
Fetal hemoglobin (g/100ml)	9.71 ± .59	10.5 ± .31

[a]All values represent means ± standard errors.

TABLE 4.3. COMPARISON OF IRON CONTENT OF THE FETAL LIVER AND PLACENTA FROM CONTROL AND TREATMENT GILTS[a]

Treatment	N[b]	Liver		Placenta	
		Iron concentration (mg/g dry tissue)	Total iron (mg/liver)	Iron concentration (mg/g dry tissue)	Total Iron (mg/placenta)
Control	31	1.29 ± .09	3.73 ± .20	.42 ± .01	5.98 ± .32
Treatment	40	1.19 ± .06	3.51 ± .18	.44 ± .03	5.19 ± .32

[a]All values represent means ± standard errors.

[b]N = number of observations.

No treatment effects were observed in fetal liver iron content. These data are in contrast to earlier findings (Ducsay, 1977) which demonstrated a significant treatment effect on total liver iron content. No explanation for this discrepancy is available.

Placenta. No differences were observed in iron content (Table 4.3) or weight (Table 4.2) of placentae from control and iron treated gilts.

Allantoic fluid. Allantoic fluid volume and iron content are summarized in Table 4.4. Although differences between the two treatment groups were not observed, the same general trend as previously reported (Ducsay, 1977) was detected. Total allantoic fluid iron from treated gilts was five times that for control gilts. Failure to detect a significant treatment effect may be due to the high degree of variation normally found when examining allantoic fluid components.

Acid Phosphatase

Earlier studies by Ducsay (1977) postulated that observed increases in allantoic fluid iron content from iron treated gilts were due to increase in uteroferrin content. Acid phosphatase activity, however, was not measured. Results from the present study indicated treatment had no effect on acid phosphatase activity in allantoic fluid or placental tissue (Table 4.5).

TABLE 4.4. COMPARISON OF ALLANTOIC FLUID VOLUME AND IRON CONTENT FROM CONTROL AND TREATMENT GILTS[a]

Treatment	N[b]	Allantoic Fluid			
		Volume (ml)	N[b]	Iron concentration (µg/ml)	Total iron (µg/allantoic sac)
Control	35	54.97 ± 13.49	30	5.11 ± .54	204.10 ± 31.38
Treatment	42	132.85 ± 31.12	22	7.13 ± 1.50	1326.54 ± 499.35

[a]All values represent means ± standard errors.

[b]N = number of observations.

TABLE 4.5. COMPARISON OF ALLANTOIC FLUID AND PLACENTAL ACID PHOSPHATASE ACTIVITY[a]

Treatment	N[b]	Allantoic fluid		N[b]	Placenta	
		Acid phosphatase concentration (μmol Pi/min/ml)	Total acid phosphatase (μmol Pi/min/ allantoic fluid sac x 10^{-3}		Acid phosphatase concentration (μmol Pi/min/g) tissue)	Total acid phosphatase (μmol Pi/min/ placenta x 10^{-3}
Control	33	2737.43 ± 739.89	64.38 ± 18.71	28	55.95 ± 5.39	9.94 ± .99
Treatment	33	845.27 ± 256.36	52.25 ± 11.05	30	56.53 ± 6.85	10.27 ± 1.60

[a]All values represent means ± standard errors.

[b]N = number of observations.

Uteroferrin

As indicated in Chapter III, over 94% of the acid phosphatase activity in allantoic fluid is due to the presence of uteroferrin (Schlosnagle et al., 1974). This same relationship has not been established for placental tissue extracts. Therefore, uteroferrin levels in placental tissue were quantified by specific radioimmunoassay for uteroferrin described in Chapter III. Placental uteroferrin concentration from iron treated gilts was significantly higher ($P<.05$) than for the control group (Table 4.6). A similar trend was observed for total placental uteroferrin content, but a treatment effect was not detected. This is an interesting finding in light of the results discussed in Chapter III. The placenta appears to store uteroferrin during the latter stages of gestation. By supplementing gilts with iron during midgestation, when endometrial uteroferrin production is maximal, increased storage of uteroferrin was observed on day 90 of gestation. However, no differences were observed in placental iron content.

TABLE 4.6. COMPARISION OF PLACENTAL UTEROFERRIN CONTENT FROM CONTROL AND TREATMENT GILTS[a]

Treatment	N[b]	Uteroferrin concentration[c] (mg/g tissue)	Total uteroferrin (mg/placenta)
Control	28	1.56 ± .15	287.31 ± 26.81
Treatment	26	2.42 ± .19	426.57 ± 46.02

[a]All values represent means ± standard errors.

[b]N = number of observations.

[c]Least squares analysis of variance indicated significant ($P < .05$) treatment effects.

CHAPTER V

TRANSFER OF RADIOLABELED IRON FROM THE MATERNAL CIRCULATION TO THE PRODUCTS OF CONCEPTION IN PREGNANT AND UNILATERALLY PREGNANT GILTS

Introduction

Seal et al. (1972) demonstrated that the rate of placental iron transport is dependent upon placental type. Species with hemochorial placentae demonstrate a rate of iron transport which is 50 times faster than that observed in species with epitheliochorial placentae. Hoskins and Hansard (1964b) examined both the rate of maternal to fetal iron transport in swine and the distribution of labeled iron in fetal tissues. The total amount of ^{59}Fe absorbed by the products of conception increased throughout gestation while the largest increase in activity in the placenta occurred between days 35 and 70 of gestation.

It has been suggested that nutrient transfer in pregnant gilts is via the embryotrophic route, i.e., by uterine secretions (Palludan et al., 1969). After labeling the circulating iron pool of a pregnant gilt with ^{55}Fe, autoradiographic examination of the uterus revealed the highest concentration of radioactive granules associated with the uterine glands and their secretions. Iron present in uterine secretions occurred in a nondialyzable form,

indicative of a protein-bound form of iron. This study confirmed earlier work of Wislocki and Dempsey (1946) which demonstrated the presence of iron in the glandular epithelium and uterine lumen by histochemical techniques.

The principal goal of this study was to examine the transfer of radiolabeled iron from the maternal circulation to tissues and fluids of the fetal-placental unit (conceptus). Specific emphasis was placed on the form in which iron is transferred. An effort was made to determine if iron present in the maternal circulation is transferred to the feto-placental unit bound to uteroferrin.

Materials and Methods

Experimental Design

Thirteen gilts of similar age, weight and genetic background were checked twice daily for estrous behavior using mature boars. The gilts were bred at 12 and 24 hours after onset of estrus and eight gilts were randomly assigned to treatment on either day 30, 60, 90, or 105 of gestation. Two gilts were assigned to treatment on days 60 and 65, and the remaining three gilts were rendered unilaterally pregnant and assigned to treatment on day 60 of gestation.

Procedure

Pregnant gilts. On the assigned day of treatment, 100 μCi ^{59}Fe (Amersham) was injected through a polyvinyl

catheter into an ear vein of each gilt. The syringe and catheter were then flushed with 5 ml of sterile saline. A maternal blood sample from a vein of the opposite ear was taken immediately after injection at a time designated as T_0. After 24 hours, the gilts were hysterectomized and fetal tissues and fluids were collected as previously described. A maternal blood sample was also obtained and designated as T_{24}. In addition to the fetal liver, placenta, allantoic and amniotic fluid, additional samples were collected. A sample of fetal blood was obtained from the umbilical cord with a 22 gauge needle attached to a 1cc syringe. The fetal spleen, left kidney and a section of right tibia were dissected out and weighed. Random samples of uterine endometrium were also collected. One of the day 60 gilts was laporotomized at 3 hours and 9 hours prior to hysterectomy. During this procedure, allantoic fluid samples were obtained from four fetuses. This was accomplished by passing a 22 gauge needle through the uterine wall into each allantoic sac. Correct needle placement was assured by digital manipulation. Samples were obtained near the end of each allantoic sac, away from the amnion.

Unilaterally pregnant gilts. Three gilts were bred and rendered unilaterally pregnany on day 18 by the method of Basha et al. (1980). At laparotomy, an 18 gauge needle attached to a 20cc syringe was inserted through the uterine wall of one of the uterine horns near the cervix. Twenty

milliliters of sterile saline were introduced into the uterine lumen, and the bolus of fluid was massaged toward the oviduct. A small incision was made in the uterine horn near the tubo-uterine junction and the embryos were flushed out of the uterine horn. A ligature was placed around the uterine horn near the junction of the uterine horn and body. The incision was closed, the uterus returned to the body cavity and the gilts were allowed to recover. On day 60 of gestation, the gilts were injected with 100 μCi ^{59}Fe and 24 hours later hysterectomized as previously described. The nonpregnant uterine horn was flushed with 80 ml of sterile 0.9% saline and the uterine flushing recovered. Tissues from the pregnant horn were collected as described previously. Endometrial tissue samples were collected from both the gravid and nongravid uterine horns.

Radioactivity Determination

Tissues. Samples of spleen, bone and kidney were placed in 13x100 mm culture tubes and counted in a gamma counter (Packard, 5130). Liver, placenta and endometrial tissues were minced, and representative samples of each tissue were weighed and placed in 13x100 mm tubes for counting in duplicate. All tissues were counted and blank tubes were included for determination of background radiation.

Fetal fluids. One milliliter aliquots of both allantoic and amniotic fluid were pipetted into 13x100 mm

culture tubes and counted. Pooled allantoic fluid samples were lyophylized and reconstituted with deionized water to one-tenth the original volume. One milliliter aliquots of pooled samples were counted, then mixed with an equal volume of carboxymethyl cellulose (CMC, Pharmacia), a cation exchanger, and incubated at 4 C for 2 hours. The samples were centrifuged at 2500 rpm for 10 minutes and the supernate recovered and counted. The pellet was washed in 10mM Tris pH 8.0, resuspended, spun at 2500 rpm for 10 minutes, the supernate discarded and the pellet counted. Afterward, the pellet was washed in 1M NaCl, 10mM Tris. The sample was spun at 2500 rpm, the supernate discarded and the pellet recovered and counted to determine if treatment with high salt buffer would remove the counts previously associated with the CMC.

Blood samples. Aliquots of whole blood from fetuses and gilts were counted. Samples were centrifuged at 3000 rpm for 10 minutes, and serum was separated and placed in separate tubes for counting. The remaining red blood cells from each sample were washed in cold normal saline, centrifuged and the supernatant poured off. The process was repeated and the cells were resuspended in 1 ml of normal saline and counted.

Uterine flushings. After collection, uterine flushings were centrifuged at 15,000 rpm for 20 minutes to remove cellular debris. A volume of the supernate from each flush equivalent to 1000 cpm (approximately 3ml) was loaded on a

Sephacryl S-200 column utilizing a .02 M sodium barbital buffer system, pH 8.0. One milliliter fractions were collected utilizing an automated fraction collector (Gilson) and counted. Protein concentration was determined for each fraction utilizing the method of Lowry et al. (1951) protein determination (Appendix A).

Ouchterlony Immunodiffusion Analysis

Coincidental peaks of radioactivity and protein were examined for the presence of uteroferrin and/or transferrin by utilizing Ouchterlony immunodiffusion plates with specific antiserum prepared against uteroferrin and transferrin. Immunological identity was indicated by the formation of precipitin lines.

Statistical Analysis

Data were analyzed by the method of least squares analysis of variance employing Statistical Analysis System (Barr et al., 1979). The pattern of ^{59}Fe accumulation in tissues and fluids as well as changes in tissue weights was examined over days of gestation relative to variation among gilts within day of gestation. Expected mean squares in the analysis of variance of the data are found in Table 5.1. Prediction equations based on least squares regression analyses are presented in Appendix B.

TABLE 5.1. ANALYSIS OF VARIANCE EXPECTED MEAN SQUARES

Source	df	EMS
Day	3	$\sigma^2_e+K_2\sigma^2_{G(D)}+K_3\sigma^2_D$
Gilt (Day)	5	$\sigma^2_e+K_1\sigma^2_{G(D)}$
Residual	N-8	σ^2_e

Results and Discussion

Tissue Weights

The changes in weight of fetal liver (Table 5.2) and placenta (Table 5.3) were significantly affected by day of gestation and were similar to those reported in Chapter III. Weight of the fetal spleen, first measured on day 60, increased significantly ($P < .01$) as gestation progressed (Table 5.2). These data are consistent with that of Ullrey et al. (1965) and Robinson (1976) who examined alternations in weight of fetal organs as gestation progressed. Similar results were observed when kidney weight was examined (Table 5.4). No total weight measurement was made on fetal bone (specifically the left tibia) since the section examined did not always represent the entire bone.

Accumulation of ^{59}Fe

Liver. The total amount of ^{59}Fe accumulated by the fetal liver from the maternal circulation after 24 hours is

TABLE 5.2. CHANGES IN WEIGHT AND TOTAL ^{59}FE CONTENT OF THE FETAL LIVER AND SPLEEN AT VARIOUS STAGES OF GESTATION[a]

Day of gestation	N[b]	Liver weight[c] (g)	Total liver ^{59}Fe (cpm)	Spleen weight[c] (g)	Total spleen ^{59}Fe[d] (cpm)
30	23	.31 ± .01	1809 ± 267	---	---
60	16	5.96 ± .51	56551 ± 8083	.07 ± .01	456 ± 57
90	22	17.07 ± .84	72972 ± 3440	.78 ± .04	5388 ± 269
105	16	19.45 ± .86	61193 ± 3596	.85 ± .05	5176 ± 498

[a]All values represent means ± standard errors.

[b]N = number of observations.

[c]Least squares analysis of variance indicated significant ($P < .01$) day effects.

[d]Least squares analysis of variance indicated significant ($P < .05$) day effects.

TABLE 5.3. CHANGES IN PLACENTAL WEIGHT AND ^{59}FE ACCUMULATION AT VARIOUS STAGES OF GESTATION[a]

Day of gestation	N[b]	Placental weight[c] (g)	Total placental ^{59}Fe (cpm)
30	23	29.0 ± 2.1	11591 ± 7915
60	16	162.8 ± 18.3	35151 ± 5540
90	22	187.4 ± 16.7	33536 ± 3973
105	16	172.7 ± 14.0	27004 ± 1617

[a]All values represent means ± standard error.

[b]N = number of observations.

[c]Least squares analysis of variance indicated significant ($P < .05$) day effects.

TABLE 5.4. CHANGES IN FETAL KIDNEY WEIGHT AND TOTAL ^{59}FE ACCUMULATION AT VARIOUS STAGES OF GESTATION[a]

Day of gestation	N[b]	Kidney weight[c] (g)	Total kidney ^{59}Fe (cpm)
30	--	---	---
60	16	.80 ± .07	283 ± 35
90	22	3.10 ± .16	458 ± 50
105	16	3.67 ± .21	562 ± 36

[a]All values represent means ± standard errors.

[b]N = number of observations.

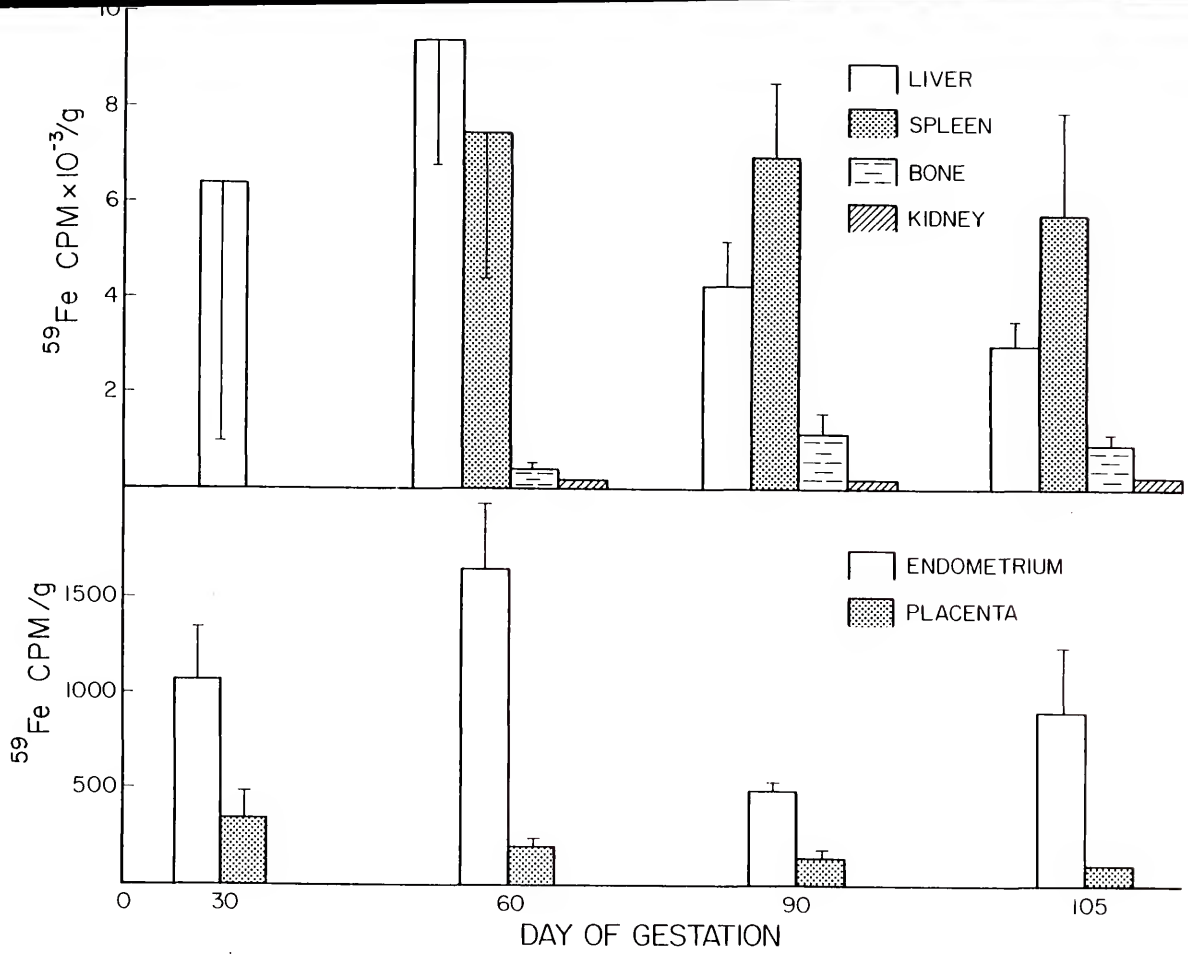
[c]Least squares analysis of variance indicated significant ($P < .05$) day effects.

demonstrated in Table 5.2. The greatest increase in total counts was observed between days 30 and 60 of gestation. This observation was expected since this is the period of time when the liver exhibits its largest increase in weight. Beyond day 60, the increase in total amount of ^{59}Fe accumulated after a single injection in the dam is relatively small. Concentration of ^{59}Fe, i.e., cpm/g wet tissue, was highest on day 60 (Figure 5.1). Although not statistically significant, the greatest accumulation of total cpm ^{59}Fe as well as ^{59}Fe concentration per gram of tissue was between days 30 and 60 of gestation. These data are in agreement with earlier studies of Hoskins and Hansard (1964b). During this period of gestation, fetal hematopoesis becomes quite active, and the fetus requires large quantities of iron. It is also interesting to note that this period in gestation is associated with maximal production and accumulation of uteroferrin. This point will be discussed further when considering ^{59}Fe accumulation in allantoic fluid.

Spleen. Unlike liver, total cpm of ^{59}Fe in spleen increased quite dramatically between days 60 and 90 of gestation (Table 5.2). This increase is due to an increase in spleen weight. Spleen weight increased from .07g at day 60 to .78g by day 90, while no substantial differences were observed in ^{59}Fe expressed as cpm/g tissue among days of gestation examined (Figure 5.1).

Bone. The concentration of ^{59}Fe determined in sections of left tibia during gestation is represented in Figure 5.1.

Figure 5.1. Concentration (cpm/g wet tissue) of ^{59}Fe in fetal tissues and endometrium 24 h after introduction of ^{59}Fe into the maternal circulation. All values represent means ± standard errors.


^{59}Fe CPM x 10^{-3}/g
10
8
6
4
2
LIVER
SPLEEN
BONE
KIDNEY
^{59}Fe CPM/g
1500
1000
500
ENDOMETRIUM
PLACENTA
0
30
60
90
105
DAY OF GESTATION

A large increase was observed between days 60 and 90 while levels remained fairly stable between days 90 and 105. The increase observed between days 60 and 90 is indicative of the shift in the major site of prenatal hematopoesis. During this period, there is a rapid proliferation of erythropoetic tissue, i.e., bone marrow. This is reflected by the large increase in the amount of labeled iron present in the bone by day 90 of gestation.

Kidney. Neither the concentration of accumulated ^{59}Fe (Figure 5.1) nor total cpm ^{59}Fe (Table 5.4) was affected by gestational age (Figure 5.1), i.e., day effects were not significant. Data for iron accumulation by fetal spleen and kidney support earlier work of Hoskins and Hansard (1964b). The authors measured levels of .009 percent and .008 percent of the total injected dose of ^{59}Fe in fetal spleen after 48 hours on days 70 and 105 of gestation, respectively. Values of .007 and .008 percent of the total injected dose were calculated for the fetal kidney on days 75 and 105 of gestation, respectively.

Fetal blood. Levels of ^{59}Fe in fetal blood are listed in Table 5.5. The amount of radiolabeled iron in whole fetal blood was maximal on day 60 and declined to days 90 and 105. A similar trend was observed with plasma and red blood cells. The major proportion of counts in whole blood were in the erythrocytes, indicating incorporation of transferred iron into fetal hemoglobin. It is interesting to note that peak levels of ^{59}Fe transferred to the fetal

TABLE 5.5. ACCUMULATION OF ^{59}FE IN FETAL WHOLE BLOOD, PLASMA AND ERYTHROCYTES AT VARIOUS STAGES OF GESTATION[a]

Day of gestation	N[b]	Whole blood (cpm/ml)	Plasma (cpm/ml)	Cells (cpm/ml resuspended cells)
30		---	---	---
60	16	3349 ± 436	275 ± 52	964 ± 171
90	22	1479 ± 52	135 ± 15	588 ± 48
105	16	1123 ± 97	106 ± 39	591 ± 37

[a]All values represent means ± standard errors.

[b]N = number of observations.

circulation are found on day 60 of gestation. As mentioned previously, this is the period of gestation when uteroferrin reaches peak levels. Although high levels of ^{59}Fe present in fetal blood at this stage of gestation may be due to increased erythropoesis, it is suggestive of a relationship between uteroferrin levels and iron transport.

Palludan et al. (1969) determined that a minimum time of 2 to 3 hours are required before radiolabeled iron from the maternal circulation was demonstrated in fetal blood, specifically plasma. A few hours later, radioactivity levels comparable to or higher than those achieved in plasma were reached in fetal erythrocytes. Further in vitro studies indicated rapid incorporation of transferrin iron into fetal erythrocytes.

Maternal blood. Changes in maternal blood levels of ^{59}Fe are summarized in Table 5.6. The majority of counts at T_0 were observed in the plasma fraction. The erythrocyte fraction contained the major portion of counts at T_{24}. This difference is presumably due to the incorporation of ^{59}Fe into maternal erythrocytes, i.e., hemoglobin, over 24h. The counts per ml of whole blood at T_0 are higher than levels at T_{24}. Aside from incorporation into maternal erythrocytes, a portion of this decline in plasma must be attributed to uptake by maternal tissues. Although no maternal tissues other than endometrium were sampled, previous studies by Hoskins and Hansard (1964b) revealed rapid incorporation of labeled iron into maternal liver and

TABLE 5.6. LEVELS OF ^{59}FE IN MATERNAL WHOLE BLOOD, PLASMA AND ERYTHROCYTES AT T_0 AND T_{24} AT VARIOUS STAGES OF GESTATION[a]

Day of gestation	Time	Whole blood (cpm/ml)	Plasma (cpm/ml)	Cells (cpm/ml resuspended cells)
30	T_0	4113 ± 411	6635 ± 1243	133 ± 130
	T_{24}	1453 ± 444	740 ± 231	2241 ± 1893
60	T_0	6096 ± 1800	10144 ± 4994	164 ± 103
	T_{24}	1235 ± 795	560 ± 519	1346 ± 594
90	T_0	3881 ± 1539	7929 ± 1240	167 ± 24
	T_{24}	1468 ± 546	281 ± 80	982 ± 81
105[b]	T_0	---	---	---
	T_{24}	---	---	---

[a]All values represent means ± standard errors.

[b]Samples hemolyzed.

spleen. This is an important factor to consider when attempting to determine the amount of ^{59}Fe actually available to the conceptus from the maternal circulation.

Endometrium. The concentration of ^{59}Fe found in maternal endometrial samples was maximal between days 30 and 60 of gestation (Figure 5.1). Earlier studies (Hoskins and Hansard, 1964b; Palludan et al., 1969) examining placental transfer of iron failed to examine isotope levels in endometrial tissue despite the fact that it is in direct association with the placenta and is, therefore, intimately involved in the process of iron transfer.

The period of gestation between days 30 and 60 is associated with maximal uteroferrin production by the endometrium (Basha et al., 1979). Iron concentration is also comparatively high during this time. These facts coincide with data on ^{59}Fe accumulation by the endometrium between days 30 and 60 (Figure 5.1). These data again imply an involvement of uteroferrin in maternal to fetal iron transport.

Allantoic and amniotic fluid. It was postulated that administration of ^{59}Fe to the dam would result in accumulation of radiolabeled iron in allantoic fluid. However, when 1 ml aliquots of allantoic and amniotic fluid were counted, no significant counts above background were detected. This was puzzling since uteroferrin has previously been demonstrated to move across the placenta (Chen et al., 1975) and to be sequestered in allantoic fluid (Bazer et al.,

1975). There may be a number of ways to explain these results. The rate of iron incorporation into uteroferrin as well as the turnover rate of uteroferrin in the allantoic fluid is not well defined. By sampling 24 hours after the injection of ^{59}Fe into the maternal circulation, the peak of ^{59}Fe in allantoic fluid may have been missed.

In an effort to circumvent this problem, one gilt was injected with 100 μCi ^{59}Fe on day 60 of gestation. Sequential allantoic fluid samples were collected from four fetuses at 3, 9, and 24 hours after injection. Another gilt was injected with 100 μCi ^{59}Fe on day 60, and allantoic fluid samples were collected on day 65 of gestation. The purpose of this sampling period was to determine if there was a longer term accumulation of ^{59}Fe in allantoic fluid 5 days after injection. No detectable levels of radioactivity were observed in small volumes of allantoic fluid samples from either of the gilts. Tissue iron levels in the conceptuses were comparable to those previously determined for day 60 of gestation and will not be discussed. It is of interest, however, that fetal tissue levels 24 hours after isotope administration were similar to levels found 5 days after ^{59}Fe injection. This fact indicates that the majority of radio-iron was transferred across the placenta during the first 24 hours after injection.

One factor not considered was the possibility of dilution of the label in allantoic fluid. If a relatively small amount of ^{59}Fe was present in allantoic fluid, in a

free form or bound to uteroferrin, the large amount of fluid present would dilute it sufficiently to mask its presence in small samples. Unilaterally pregnant gilts were utilized to overcome the problem of dilution of uterine secretions in the allantoic fluid. Actual secretory products from the maternal endometrium, comparable to those being produced by the contralateral gravid horn (Basha et al., 1980), are sequestered in the uterine lumen. The secretions can then be recovered by the flushing technique previously described.

Acid phosphatase activity and protein content of flushings from day 60 unilaterally pregnant gilts are summarized in Table 5.7. Both protein and acid phosphatase levels are comparable to those observed in an earlier study (Basha et al., 1980). Schlosnagle et al. (1974) indicated that 1 mg of uteroferrin is capable of hydrolyzing approximately 100 μmole to 300 μmole p-nitrophenylphosphate/min. Based on this calculation, uterine flushings from the day 60 nongravid uterine horns would contain a minimum of 545 mg of uteroferrin.

Total counts and counts per ml of uterine flushing are presented in Table 5.7. Although the levels are not strikingly high, significant counts were observed in the uterine flushings. A specific activity of approximately 14 cpm/mg protein was observed. In order to determine if any of the radioactivity was associated with uteroferrin, a 3 ml sample of each flush was loaded on a Sephacryl S-200 column as described in Materials and Methods.

TABLE 5.7. ACID PHOSPHATASE ACTIVITY, PROTEIN CONTENT AND ^{59}FE CONTENT FROM UTERINE FLUSHINGS PER HORN FROM THE NONGRAVID HORN OF UNILATERALLY PREGNANT GILTS[a]

No. of flushings	3
Acid phosphatase (mol Pi/min/ml)	1,008 ± 293
Total acid phosphatase (mol Pi/min/flush)	54,416 ± 23,801
Protein conc. (mg/ml)	17.0 ± 1.0
Total protein (mg)	855 ± 170
^{59}Fe conc. (cpm/ml)	232 ± 28
Total ^{59}Fe (cpm)	11,557 ± 2,218

[a]All values represent means ± standard errors.

A representative elution profile is demonstrated in Figure 5.2. Two major protein peaks were observed at fractions 81 and 95. Both protein peaks co-chromatographed with peaks of radioactivity. The first peak had an elution volume similar to that of transferrin, while the second peak eluted at a volume similar to that for uteroferrin. The column was standardized previously with ^{125}I transferrin and ^{125}I uteroferrin. The peak elution volumes for these proteins are indicated in Figure 5.2 by Tf and Uf, respectively.

In order to positively identify the proteins under these peaks which co-chromatographed with peak ^{59}Fe levels, Ouchterlony immunodiffusion analysis was employed. Fractions 81 and 95 were tested against antiserum specific for porcine uteroferrin and transferrin. Fraction 76 was also included since it represented a leading shoulder to the protein peak where transferrin normally elutes and was associated with some radioactivity. All three fractions formed a single precipitin line with the Uf antiserum (Figure 5.3). When the same fractions were tested against Tf antiserum (Figure 5.4), neither fraction 81 nor 95 demonstrated any cross-reactivity. Fraction 76 demonstrated a relatively imperceptable precipitin band, indicating small amounts of transferrin present in this fraction.

All three fractions examined demonstrated the presence of immunologically recognizable uteroferrin. Based on the column calibration, uteroferrin normally elutes in the area

Figure 5.2. Sephacryl S-200 chromatorgraphy of a uterine flushing collected from a unilaterally pregnant gilt injected with ^{59}Fe. Protein concentration (---) and ^{59}Fe content (——) are plotted against elution volume (1 ml fractions). The column was previously calibrated with purified transferrin (TF) and uteroferrin (UF). Expected elution volumes of these proteins are indicated by arrows.

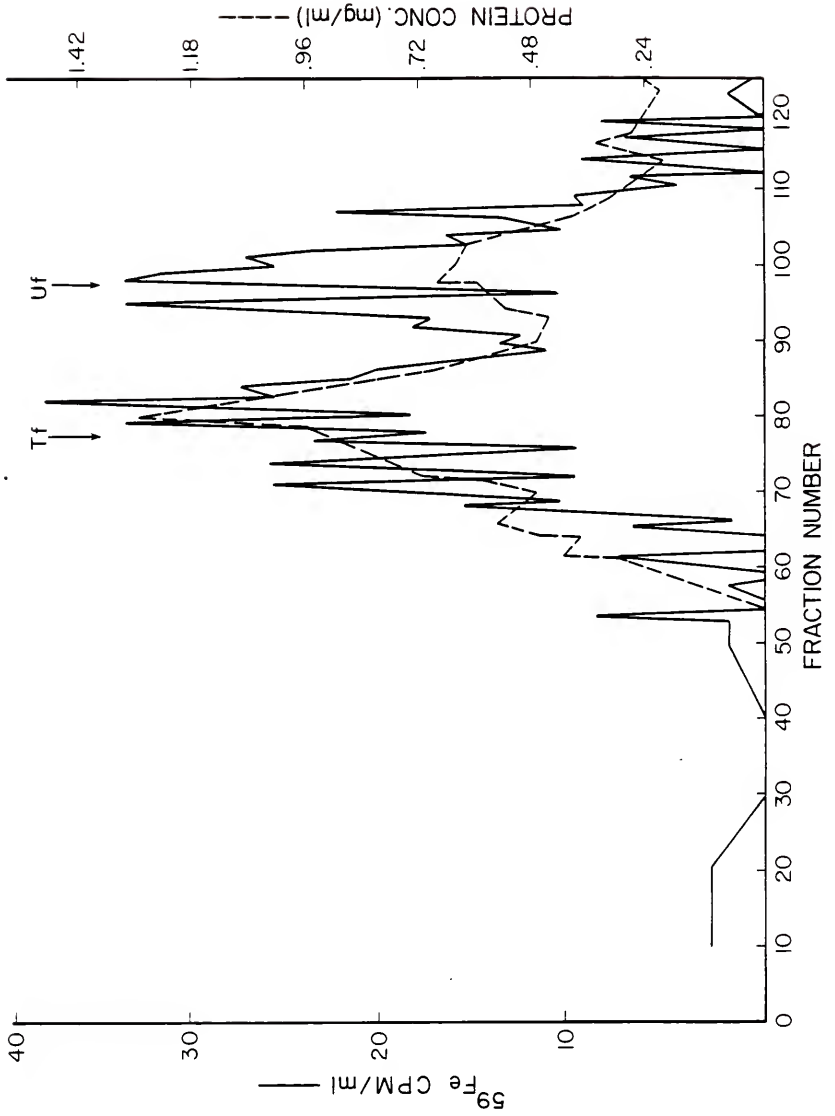

PROTEIN CONC. (mg/ml) ----
1.42
1.18
.96
.72
.48
.24
Uf
Tf
59Fe CPM/ml ——
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
110
120
FRACTION NUMBER

Figure 5.3. Ouchterlony immunodiffusion of protein fractions (see Figure 5.2) eluted from a Sephacryl S-200 column loaded with uterine flushing from a day 60, unilaterally pregnant gilt. Uteroferrin antiserum (AUF) was placed in the center well. Wells labeled 76, 81 and 95 represent those respective protein fractions and the well labeled UF represents uteroferrin standard. Single precipitin lines formed against protein in fractions 76, 81 and 95 indicated the presence of uteroferrin in each of the fractions.

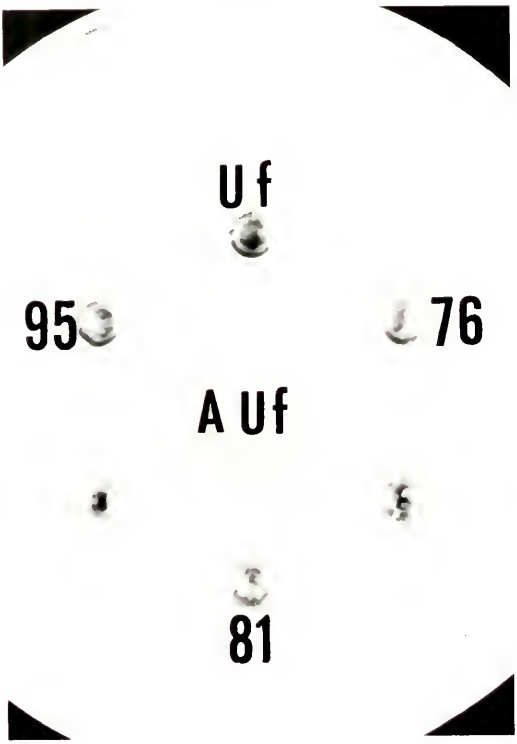
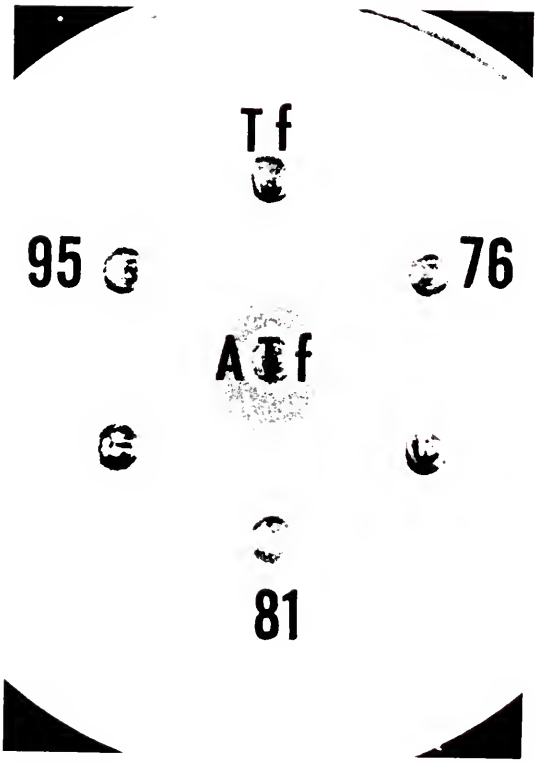

Uf
95
76
A Uf
81

Figure 5.4. Ouchterlony immunodiffusion of protein fractions (see Figure 5.2) eluted from a Sephacryl S-200 column loaded with uterine flushing from a day 60, unilaterally pregnant gilt. Transferrin antiserum (ATF) was placed in the center well. Wells labeled 76, 81 and 95 represent those respective protein fractions and well labeled TF represents transferrin standard. No precipitin lines were formed against protein in any of the fractions which is indicative of the absence of transferrin.


Tf
95
76
Tf
81

of fraction 95 corresponding to a molecular weight range of approximately 32,000 to 35,000. The protein from the other two peaks that cross-reacted with uteroferrin antiserum may be a dimer of uteroferrin or an association with some other protein that formed under the buffer conditions employed in the gel filtration chromatography. These data indicate that the major iron-containing secretory protein of the endometrium is uteroferrin and not transferrin. The major levels of ^{59}Fe were associated with uteroferrin in the uterine secretions.

Iron levels in the fetal tissues collected from the contralateral pregnant horn were comparable to levels observed previously in the day 60 bilaterally pregnant gilts and will, therefore, not be discussed. However, due to the lack of success in isolating ^{59}Fe uteroferrin from allantoic fluid, allantoic fluid from the three unilaterally pregnant gilts was utilized.

The pooled allantoic fluid samples were concentrated 10x and 1 ml aliquots were mixed with CMC and counted. Counts per ml of concentrated fluid are listed in Table 5.8. After washing, the majority of the counts remained bound to CMC and were designated the CMC + fraction. The counts were subsequently removed by high salt Tris buffer. Uteroferrin is a CMC + protein (Schlosnagle et al., 1974; Bazer et al., 1975). Data in Table 5.7 indicate that radiolabeled iron present in concentrated allantoic fluid is associated entirely with a basic protein which is most probably

TABLE 5.8. LEVELS OF ^{59}FE IN CONCENTRATED ALLANTOIC FLUID FROM DAY 60 UNILATERALLY PREGNANT GILTS[a]

No. of samples	3
cpm/ml	29 ± 11
CMC + fraction (cpm)	20 ± 10
Counts remaining after high salt wash (cpm)	2 ± 2

[a]Values represent means ± standard errors.

uteroferrin. The inability to demonstrate the presence of ^{59}Fe in unconcentrated allantoic fluid of pregnant gilts during gestation may be due to a dilution effect. Allantoic fluid may also serve as a "spillover" area. Uteroferrin may release iron directly at the level of the placenta, and excess uteroferrin might accumulate in the allantoic fluid pool.

Data from the unilaterally pregnant uterine flushings reveal that the major iron-containing protein produced by the maternal endometrium is uteroferrin. The inability to demonstrate the presence of radiolabeled uteroferrin in allantoic fluid appears to be due to a simple dilution of the protein in a large volume of fluid. It has been postulated that iron transported to the allantoic by uteroferrin is available for uptake by the allantoic epithelium (Bazer et al., 1975; Ducsay, 1977). Based on this study, uteroferrin may also supply iron directly to the placenta. Twenty-four hours after ^{59}Fe injection, substantial levels of ^{59}Fe are present in the feto-placental unit, while very small levels of the label are found in allantoic fluid. The concept of macromolecular uptake by the allantoic epithelium is discussed in Chapter VI.

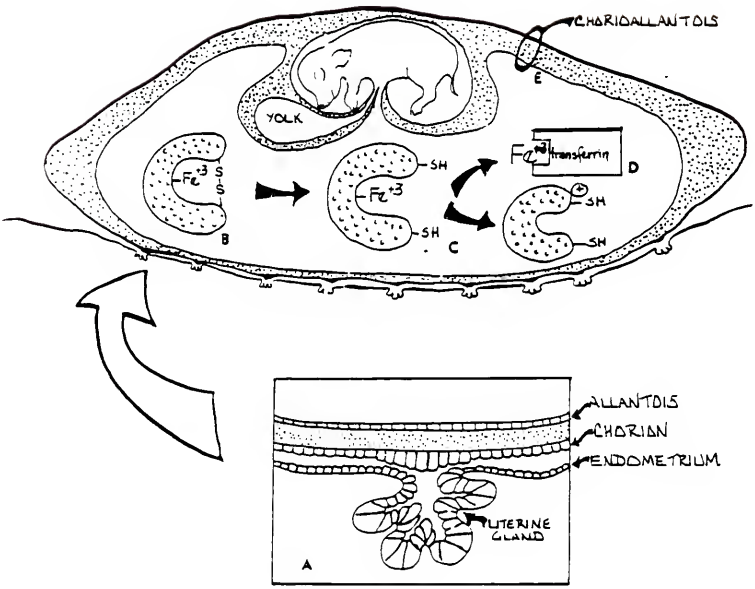
CHAPTER VI
IN VITRO AND IN VIVO ABSORPTION OF MACROMOLECULES BY THE PORCINE ALLANTOIS

Introduction

The proposed mode of uteroferrin-mediated iron transport is dependent upon the ability of allantoic epithelium to absorb iron or protein-bound iron compounds present in allantoic fluid. The model of this proposed mechanism is illustrated in Figure 6.1. Once uteroferrin reaches the reducing environment of the allantoic fluid, or possibly the chorioallantoic membrane, iron bound to the protein is released and is bound by transferrin. Since the allantois arises as a diverticulum of the fetal hind gut and is therefore lined with gut endoderm, it seems reasonable to postulate that allantoic epithelium has absorptive capabilities.

Protein-bound iron present in allantoic fluid may be absorbed in a manner similar to the mechanism of gamma globulin uptake observed in the neonatal gut (Lecce, 1966; Clarke and Hardy, 1971). The authors indicated that the most likely mode of uptake is pinocytosis. More recent studies (Brown and Goldstein, 1979) demonstrated that uptake of certain large molecules such as lipoproteins into absorptive cells may involve receptor-mediated endocytosis.

Figure 6.1. Proposed model of uteroferrin-mediated iron transport in swine. Uteroferrin, produced by uterine glands (A) is transported across the placenta and sequestered in allantoic fluid (B). Under the reducing environment of allantoic fluid, iron is released from uteroferrin (C) and bound by transferrin (D). Iron present in this form is absorbed by allantoic epithelium and made available to the developing conceptus (E).


CHORIOALLANTOIS
E
YOLK
Fe^{+3}
S
S
B
SH
Fe^{+3}
SH
C
Fe^{+3}
transferrin
D
SH
SH
ALLANTOIS
CHORION
ENDOMETRIUM
UTERINE GLAND
A

This experiment was designed to examine the absorptive capacity of the allantoic epithelium with particular emphasis on macromolecular uptake. By utilizing fluorescein-tagged proteins, it was possible to monitor tissue uptake as demonstrated by Lecce (1966). Active pinocytosis by the allantoic membrane may be an integral part of the uteroferrin mediated iron transport mechanism permitting iron uptake from allantoic fluid.

Materials and Methods

Experimental Design

A total of eight crossbred gilts of similar age, weight and genetic background were utilized for the various aspects of this study. The gilts were checked twice daily for estrous behavior using mature boars and were bred at 12 and 24 hours after onset of estrus. On either day 60 or 61 of gestation each gilt was assigned to surgery for the collection of placental tissue.

Preparation of Fluorescent Proteins

Gamma globulin (FγG). Fifty milliliters of 4% γ globulin (United States Biochemical) in 0.5M carbonate-bicarbonate buffer, pH 9.0, was combined with 1g fluorescence isothiocyanate (10% on cellite) (United States Biochemical) and stirred 16 hours at 4 C. The solution was centrifuged at 5,000 rpm for 15 minutes and the supernate

dialyzed against 10mM Tris buffer, pH 8.0, at 4 C for one week with daily buffer changes.

Uteroferrin (FUF). Purified uteroferrin was combined with fluroescein isothiocyanate (FITC) in a 2:1 ratio and treated as previously described for the preparation of FγG.

Transferrin (FTF). Fluorescein-tagged transferrin was prepared in the same manner as FγG and FUF.

Procedure

In Vitro. Surgery was performed on day 60 of gestation with anesthesia induced with sodium thiopental and maintained with methoxyflurane (Pittman-Moore). The uterus was exposed by midventral laparotomy and a 3-4 cm incision was made through the uterine wall, exposing the placenta. A section of the chorioallantois was then peeled away from the underlying endometrium and placed in a sterile culture dish containing minimal essential medium (MEM) (Appendix A). The tissue was rinsed to removed excess blood and smaller sections approximately 1 cm in diameter were cut for use in the incubations. In addition, sections of amnion and fetal small intestine were obtained and treated in a similar manner.

After washing, sections of choriallantois (CA), amnion (AM) and fetal intestine (FI) were placed in individual 30 ml incubation flasks containing 5 ml MEM. They were allowed a 30 minute equilibration period in a shaker bath at 37 C under a gassing hood with an atmosphere of 95% O_2 and 5%

CO_2. At the end of the equilibration period, CA and AM tissues were transferred to fresh medium containing: 1) no protein-control; 2) FγG; 3) FUF or 4) FTF. Fetal intestine was incubated in MEM alone or with FγG. The incubation was carried out under the same conditions described for the equilibration period.

At the end of the incubation, tissue sections were washed in fresh MEM and placed in 10% buffered formalin for 16 hours. The tissues were then processed automatically through increasing concentrations of alcohol and xylene (Autotechnicon). After fixation, the tissues were blocked in paraffin and 5μ sections were cut, placed on slides and dried. The paraffin was subsequently removed and the sections were mounted in a nonfluorescent mountant (Permount). Sections of CA and AM were also incubated with FγG in an anaerobic atmosphere (N_2) or with FγG and 10^{-4} M Na-arsenate. Previously described incubation conditions and tissue preparation methods were utilized.

A modification of the in vitro method outlined above was used with a portion of the CA collected. Instead of being placed directly in an incubation flask, a section of CA was placed on a modified Ussing chamber. This consists of a lucite chamber which is filled with medium and divided by the section of placenta. A mixture of 95% O_2, 5% CO_2 was bubbled through both sides of the chamber for a 30 minute equilibration period at 37 C. At the end of this time, FγG was added to either the chorionic or the allantoic

side of the chamber to a concentration of 0.5%. The incubation was terminated after 30 minutes and the tissue sections were washed, fixed and sectioned as described earlier.

In Vivo. On day 60 of gestation the uterus was exposed via midventral laparotomy, and individual allantoic fluid sacs were isolated by digital manipulation. A 20 gauge needle attached to a 20 cc syringe was inserted through the uterine wall into the individual allantoic sacs. Correct placement was assured by insertion near the end of each allantoic sac and away from the amniotic sac and the withdrawal of a small amount of allantoic fluid into the syringe. Once needle placement was confirmed, either 20 cc of FγG (4%) or 10 cc of unlabeled porcine gamma globulin (γG) (10mg/cc) was injected into the allantoic sac. The position of the injected fetuses was noted, the uterus returned to the body cavity and the incision closed. The gilts were allowed to recover for 24 hours and were subsequently hysterectomized. Sections of CA were collected from the fetuses which had FγG injected into the allantoic sacs and fixed and sectioned. Samples of fetal and maternal blood were collected from the animals which had γG injected into the allantoic fluid compartment as well as from uninjected control fetuses. In addition, allantoic fluid volumes were measured. Blood samples were refrigerated overnight, centrifuged at 3000 rpm for 10 minutes and the serum collected and frozen at -20 C until analyzed on Ouchterlony immunodiffusion plates for the presence of γ globulin.

Photomicroscopy. Tissue sections were observed with a Zeiss microscope, utilizing a high intensity ultraviolet light source. Photographs were made on either Kodak Plus X or Kodochrome film with bracketed exposure times of 1, 2 and 3 minutes. The photographs were taken through a 10X occular and either a 27X or 54X oil objective.

Immunodiffusion. Serum samples were tested for the presence of γG by Ouchterlony agar gel immunodiffusion analysis. The center well was loaded with 20μℓ of rabbit antipig IgG (United States Biochemical) and the surrounding wells with 20μℓ aliquots of fetal serum. The plates were incubated at 4 C for 48 hours and examined for the presence of precipitin lines.

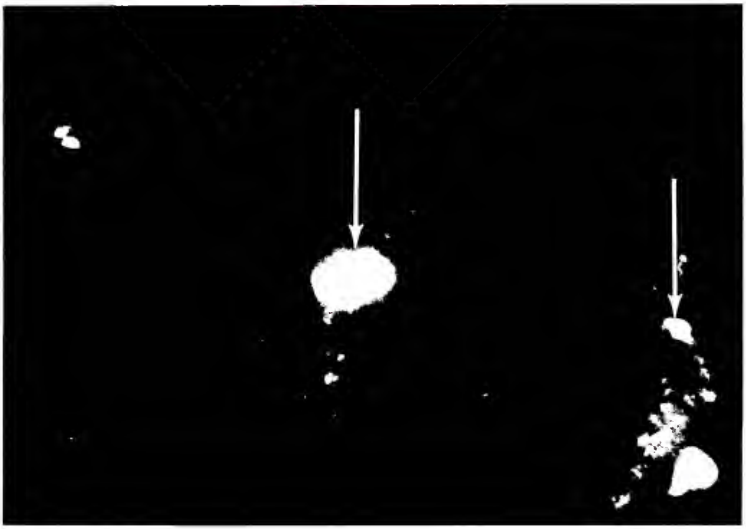
Results and Discussion

In Vitro

The fetal gut, like the neonatal gut, is capable of macromolecular absorption. This is demonstrated in Figure 6.2 by the presence of intense fluorescent droplets of FγG within the cells as indicated by the arrows. Areas of nonspecific labeling and autofluorescence are easily distinguishable from areas of direct uptake. These areas of specific uptake are in direct contrast to sections of AM incubated with FγG, FUF and FTF. Although some nonspecific fluorescence was observed, no flurorescent droplets were present within the cells of the amnion. A representative

Figure 6.2. Absorptive capacity of day 60 fetal gut incubated *in vitro* with fluorescence in labeled γ globulin (FγG) at 37 C for 30 min in MEM. Arrows point to droplets of FγG within epithelium.

Figure 6.3. Section of day 60 amnion incubated *in vitro* with fluorescein labeled γ globulin (FγG) at 37 C for 30 min in MEM. No absorption of FγG was observed.



section of amnion is illustrated in Figure 6.3. Chorion and allantois were incubated together as CA. However, observations on absorption will be discussed separately.

In examining the chorionic epithelium, it was difficult to distinguish specific uptake of fluorescein-tagged proteins. This is illustrated in Figure 6.4. This may be explained by the fact that during the process of separating the chorion from the endometrium, some cellular disruption may have taken place. This would account for the homogeneous increase in nonspecific fluorescence. However, when areas of the areolae were examined, specific uptake was observed. In Figure 6.5, FγG is indicated by the arrow. These findings are in agreement with the earlier work of Chen et al. (1975) which demonstrated the immunofluorescent localization of uteroferrin in the areolae.

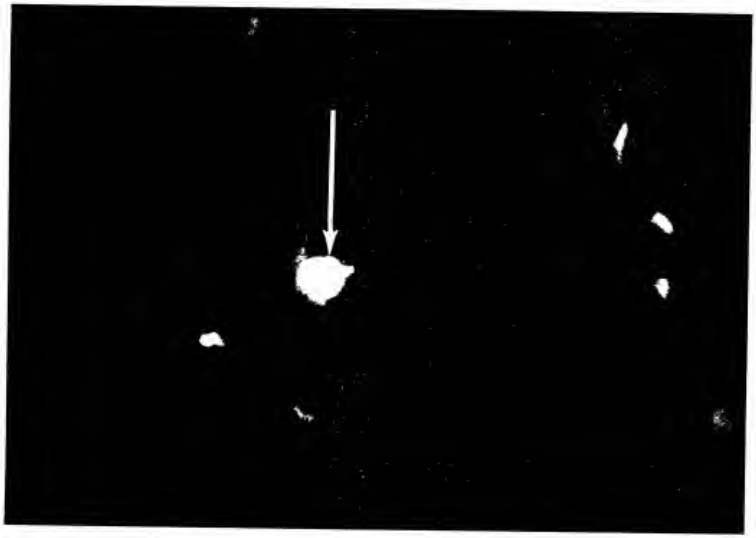
An interesting observation is that the allantoic epithelium was also demonstrated to absorb fluorescein-tagged proteins. Figures 6.6 and 6.7 illustrate uptake of FγG and FUF, respectively. Specific areas of intense fluorescence were observed in both cases (see arrows). Uptake was also observed when FTF was used in the incubation medium. Figure 6.8, although difficult to interpret, represents a section of allantois in which FTF is observable within the lumen of an arteriole present in the mesodermal tissue. There is a great deal of nonspecific fluorescence which in part is due to autofluorescence of the endodermal cells lining the blood vessel. In addition, there may be

Figure 6.4. Section of day 60 chorion incubated *in vitro* with fluorescein labeled γ globulin (FγG) at 37 C for 30 min in MEM. No specific uptake of FγG was observed.

Figure 6.5. Section of day 60 areola incubated *in vitro* with fluorescein labeled γ globulin (FγG) at 37 C for 30 min in MEM. Arrow indicates droplet of FγG within epithelium.

Figure 6.6. Section of day 60 allantoic incubated *in vitro* with fluorescein labeled γ globulin (FγG) at 37 C for 30 min in MEM. Absorption of FγG indicated by arrow.

Figure 6.7. Section of day 60 allantoic incubated *in vitro* with fluorescein labeled uteroferrin (FUF) at 37 C for 30 min in MEM. Absorption of FUF indicated by arrow.



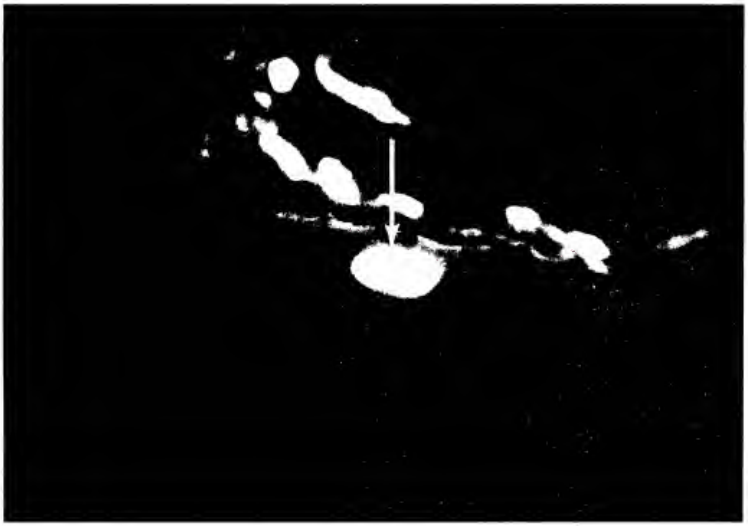


Figure 6.8. Section of day 60 allantois incubated *in vitro* with fluorescein labeled transferrin (FTF). Droplets of FTF in the lumen of an allantoic blood vessel indicated by arrows.

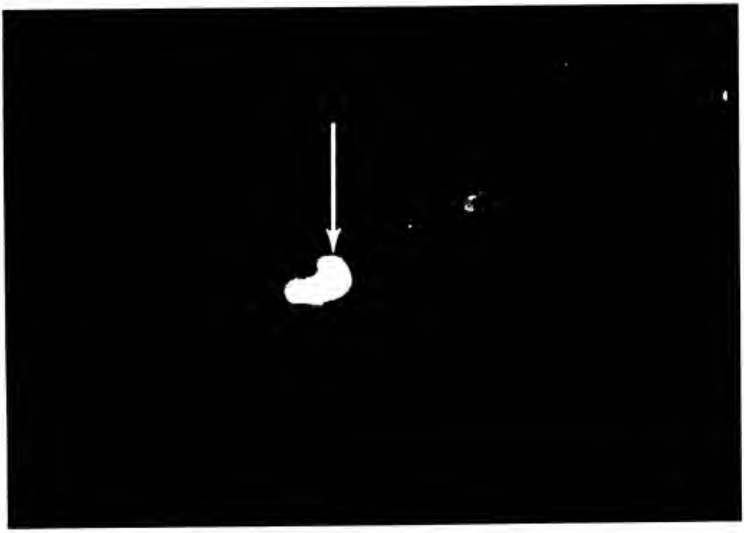
some breakdown of the labeled protein which would allow for nonspecific uptake of the FITC. However, the arrows indicate two areas of intense fluorescence which are indicative of the presence of FTF within the blood vessel. This point will be discussed further later in this section.

In order to examine the possibility of uptake being an active cellular process as opposed to simple diffusion, the effect of metabolic inhibitors was tested. Sections of CA and AM were incubated in either 10^{-4} Na-arsenate in MEM or in MEM under a N_2 atmosphere. In each case, inhibition of uptake was observed. It is not surprising that no uptake occurred in the AM sections since, even without inhibiting factors involved, no uptake was observable as described previously (Figure 6.3). Uptake of FγG was inhibited in both chorionic and allantoic tissue. A representative section of allantois demonstrating lack of uptake appears in Figure 6.9. It does appear that inhibition of an active cellular absorptive process occurred, preventing uptake of the FγG.

One question that arose during the course of this study involved the unilateral integrity of the CA. Under the incubation conditions used, the chorionic as well as the allantoic epithelium were exposed to fluorescein-tagged proteins. From the early work of Brambell (1933) and later studies by Palludan et al. (1969), and Chen et al. (1975), it is well established that the chorion is a site of absorption. Therefore, it was possible that the presence of

Figure 6.9. Effect of 10^{-4} M sodium arsenate on absorption of fluorescein labeled γ globulin (FγG) by day 60 allantois. This section of allantois was preincubated *in vitro* at 37 C with 10^{-4} M sodium arsenate in MEM for 30 min. Following preincubation, allantoic tissue was transferred to fresh MEM containing FγG for 30 min. No droplets of FγG were observed within epithelium.

Figure 6.10 Section of day 60 allantois incubated *in vitro* in an Ussing chamber with fluorescein labeled γ globulin on the epithelial side of the membrane. After incubation at 37 C in MEM, FγG was present within the epithelium (arrow).



labeled proteins in the allantoic epithelium was due to uptake and transfer by the chorionic epithelium and not the allantois. In order to circumvent this problem, a modified Ussing chamber was employed. This method permitted either side of the placenta to be exposed to FγG. Figure 6.10 represents a section of allantoic epithelium which had been incubated in the chamber with FγG present only on the allantoic side of the CA. As indicated by the arrows, FγG is present in the allantoic epithelium. Therefore, the uptake observed here, as well as in the previous in vitro incubations, can be attributed to absorption by the epithelium of the allantois and not transfer from the chorionic side of the placenta.

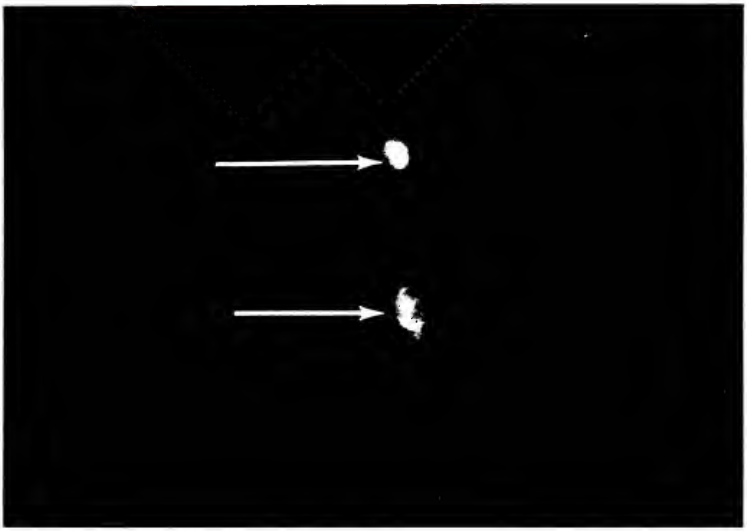
In Vivo

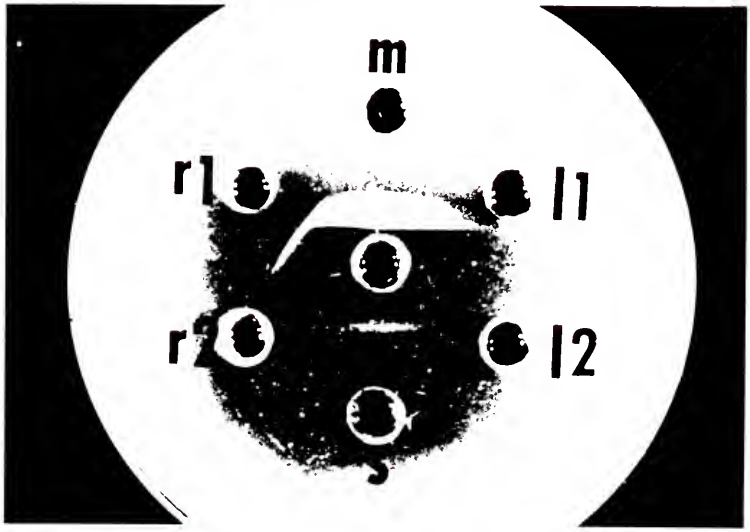
Results from this portion of the study confirm the in vitro results. The FγG injected into the allantoic fluid sacs of day 60 pregnant gilts was found to be present in the allantoic epithelium (Figure 6.11). Droplets of FγG were visible in numerous tissue sections. The in vitro incubation system appears to be a good approximation of events that occur in vivo.

The fate of macromolecules absorbed by the allantois was examined by injecting unlabeled γG into allantois fluid sacs of day 60 pregnant gilts and collecting fetal serum samples 24 hours later. A total of three gilts was used in this phase of the study. Allantoic fluid sacs of two

Figure 6.11. *In vivo* absorptive capacity of day 60 allantois. Section of allantois from a day 60 pregnant gilt which had fluorescein labeled γ globulin (FγG) injected into the allantois was obtained at hysterectomy. Arrows point to droplets of FγG within allantoic epithelium.

Figure 6.12. Ouchterlony immunodiffusion of serum from fetuses which had γ globulin introduced into allantoic fluid *in vivo* (R_1 and R_2) and uninjected controls (L_1 and L_2). The center well contains γ globulin antiserum. Maternal serum (MS) and γ globulin standard (S+D) were also tested. A single precipitin line between R1 and MS indicated the presence of γ globulin in serum from fetus R1 and maternal serum.




m
r1
l1
r2
l2

fetuses from each gilt were injected while two fetuses in the opposite uterine horn of each gilt served as controls. Of the six fetuses which had γG introduced into the allantois, two demonstrated the presence of the protein in the serum based on Ouchterlony immunodiffusion, while γG was not present in the serum from the control fetuses. Figure 6.12 represents the fetal serum samples from the injected as well as the unlabeled control fetal serum from gilt #347. Wells labeled R_1 and R_2 were loaded with fetal serum samples from fetuses that had γG injected into the allantoic fluid. A clear precipitin band was found from the fetal serum from R_1, while no response was observed in R_2. Neither of the two control serums (L_1 or L_2) exhibited a precipitin line. Although not a uniform response, it does appear that the γG injected into the allantoic fluid is absorbed by the allantoic epithelium. The absorbed protein reaches the fetal circulation totally intact or at least in an immunoprecipitable form. The combined results from this study are summarized in Table 6.1.

In conclusion, this study indicates that the epithelial lining of the allantois has absorptive properties. Thus, nutrients present in the allantoic fluid may serve as a readily available nutrient source for the developing porcine conceptus. In particular, iron transported to the allantoic fluid bound to uteroferrin may be incorporated into the fetal circulation. Based on studies by Buhi et al. (1979), it seems unlikely that the uteroferrin molecule is absorbed

TABLE 6.1. ABSORPTION OF FLUORESCENCE-TAGGED PROTEINS BY PORCINE CHORION (C), ALLANTOIS (AL), AMNION (AM) AND FETAL INTESTINE (FI) IN VITRO AND IN VIVO.

	In Vitro						In Vivo
Tissue	FγG	FUF	FTF	FγG Ussing Chamber	FγG 10^{-4}M Na Arsenate	FγG N_2 Atmosphere	FγG
C	+	+	+	+	-	-	-
AL	+	+	+	+	-	-	+
AM	-	-	-	-	-	-	0
FI	+	0	0	0	0	0	0

+ = uptake
- = lack of observable uptake
0 = not tested

from the allantoic fluid. Recent studies (W.C. Buhi, personal communication) indicate that transferrin is present in allantoic fluid which binds iron released by uteroferrin. It is the transferrin molecule that is most likely absorbed from the allantoic fluid.

In the in vivo studies in which γG was injected into the allantoic fluid, immunoprecipitable γG was observed in the serum of only two of the six fetuses treated. This inconsistency may be due to the large variation in allantoic fluid volumes of these fetuses. The fluid volume of the two fetuses in which γG was observed was lower than in the other four fetuses in the group. Since the same amount of γG was injected into each fluid sac, a greater concentration would be achieved in fetuses with lower allantoic fluid volumes. This could account for the lack of uniformity in absorption. It is important to note that γG was not observed in the serum of any of the uninjected control fetuses. This is expected since the serum profile of the neonatal pig is devoid of γG (Brambell, 1958; Lecce and Matrone, 1960).

Recent studies (Brown and Goldstein, 1979) indicated that proteins and peptides may be transported across cells by receptor mediated endocytosis. The presence of uteroferrin receptors in the chorioallantois was not examined in this study. However, recent studies (Mullins and Roberts, unpublished data; Ducsay, Roberts and Bazer, unpublished) do not indicate the presence of specific uteroferrin receptors on the pig placenta.

CHAPTER VII
GENERAL DISCUSSION

Uterine secretions play a major role in nutrition of the early embryo. Prior to implantation, the embryo lies free in the uterine lumen, bathed by endometrial secretory products. The length of time the embryo/fetus remains dependent upon uterine secretions is related to species and placental type. In species such as the pig, with epitheliochorial placentation, the fetus depends upon uterine secretions throughout gestation.

Brambel (1933) suggested the importance of uterine secretions in swine during gestation. Areolae, which form in direct apposition to the opening of uterine glands, are responsible for absorption of the secretions. Knight et al. (1977) further emphasized the importance of this concept. When the number of areolae was experimentally reduced, a significant reduction in fetal size was observed. Chen et al. (1975) provided direct evidence for areolar uptake of uteroferrin by utilizing a fluorescent antibody technique. Uteroferrin was localized in glandular maternal endometrium, and, as gestation progressed, placental areolae became highly fluorescent, indicative of uptake of uterine secretions by areolae. Bazer et al. (1975) demonstrated the presence of uteroferrin in allantoic fluid of pregnant

gilts between days 30 and 100 of gestation. No uteroferrin was present in allantoic fluid prior to day 30, which is when areolar formation begins.

Since the uteroferrin molecule contains iron, it was postulated that this glycoprotein was involved in transport of iron from the maternal system of the fetal-placental unit. One question that arose was related to actual iron content of the protein. As discussed in Chapter II, Campbell et al. (1978) reported that uteroferrin binds two iron atoms while others(Schlosnagle et al., 1974; Roberts and Bazer, 1980) reported one atom present. By atmoic absorption spectrometry, it appears that uteroferrin binds one iron molecule, i.e., it is composed of approximately .170% iron which is consistent with a molecular weight of approximately 32,000 to 35,000 (Appendix A).

The role of uteroferrin in iron transport during gestation was examined initially by Ducsay (1977). The pattern of iron accumulation in allantoic fluid was examined and appeared similar to that previously observed for allantoic fluid acid phosphatase activity, an indicator of uteroferrin content (Bazer et al., 1975). Data presented in Chapter III further illustrated this relationship. Iron accumulation and acid phosphatase activity appear to have a temporal relationship as gestation progresses. Significant increases in allantoic fluid iron content do not occur until formation of the areolae after day 30 of gestation. It is also during this period that fetal erythropoesis becomes quite

active (Wadill et al., 1962), and fetal iron needs are greatly increased.

Ducsay (1977) reported that iron injections into the dam between days 40 and 60 of gestation resulted in increased allantoic fluid iron on day 90 of gestation. Increases in fetal liver iron were also observed. By providing additional iron to the dam during the period of peak uteroferrin production, it was postulated that more iron would be transported to the developing conceptus. A similar but more extensive experiment was reported in Chapter IV. Although a similar increase in allantoic fluid iron was observed, no differences were determined between control and iron treatment group. The only difference between the present study and that previously reported (Ducsay, 1977) was the timing of the iron treatment. Iron injections in the earlier study were performed on days 40, 50 and 60 while those in the present study were on days 40, 45, 50, 55 and 60. However, total amount of iron injected was the same. It is doubtful that this slight variation in treatment protocol would account for the lack of treatment effect in the present study. Placental tissue was also examined, and uteroferrin content was significantly higher ($P<.05$) in tissue extracts from iron treated gilts compared to untreated controls. Iron treatment of the dam between days 40 and 60 of gestation does have an effect on iron content and uteroferrin levels of the fetal-placental unit. Results of these studies, however, are inconsistent. It does not appear

that this method of iron therapy will obviate the need for neonatal iron injections to prevent anemia in the neonatal pig.

The initial model of uteroferrin mediated iron transport (Bazer et al., 1975) proposed that uteroferrin, sequestered in the allantoic fluid sac, released iron. Iron, in the ferrous form, was subsequently absorbed by allantoic epithelium. Since the allantois is derived embryologically from the fetal hind gut, it is lined with gut epithelium and should therefore have absorptive capabilities. Studies by Buhi (1980) revealed that iron released from uteroferrin in allantoic fluid is then bound by transferrin.

Macromolecular uptake studies in Chapter VI demonstrated that allantoic epithelium is capable of absorbing a number of proteins ranging in size from 35,000 (uteroferrin), 78,000 (transferrin) to over 150,000 (γ globulin). It appears that iron released from uteroferrin and bound by transferrin can be incorporated into the fetal system by uptake of the intact transferrin molecule. Uptake appears to be an active cellular process, analogous to the pinocytotic macromolecular uptake observed in the neonatal gut by Lecce (1966) and Clark and Hardy (1971). Uptake does not appear to involve receptor mediated uptake (Ducsay, Roberts, and Bazer, unpublished).

An interesting observation noted in Chapter VI was the apparent uptake of γ globulin by the areolae (Figure

6.5). Transfer of passive immunity in swine does not occur during the prenatal period (Morris, 1968). Immunity is transferred via colostrum after birth. There appears to be a block to γ globulin transfer across the placenta. If γ globulin is placed on the allantoic side of the placenta, uptake occurs (Figure 6.6, 6.10 and 6.11). Immunologically recognizable γ globulin also appears in the fetal circulation (Figure 6.12). In species such as the human, placental transfer of γ globulin is dependent upon uptake by endocytotic vesicles of the syncytiotrophoblast, involving specific Fc receptors for IgG (Balfour and Jones, 1977). This is in contrast to earlier studies by Hemmings and Williams (1976) that postulated nonselective pinocytosis as the major route of entry of protein into cells. Lin (1980) indicated that human IgG is incorporated into endocytotic vesicles or phagosomes. The phagosomes fuse with lysosomes to form phagolysosomes in which some IgG escapes proteolytic digestion. The vesicles then transport IgG to the basal membrane of the syncytiotrophoblast and discharge their contents by end ocytosis. Then IgG diffuses through the interstitial space and enters the vascular lumen. A similar type of transfer does not occur in the pig. This may be due to the more complex nature of the placenta. Compared to the hemochorial placenta of the human, there are three additional tissue layers (maternal) separating the maternal and fetal circulations.

Figure 6.5 depicts uptake of γ globulin by the pig placenta. It should be remembered, however, that this is an in vitro system. The fluorescein tagged γG was placed in direct apposition to the chorionic tissue layer, with no interference by the maternal tissue layers. In the intact animal, no evidence has been presented to indicate the presence of immunoglobulins in uterine secretions of swine. If small amounts did reach the placenta, proteolytic digestion in phagosomes might prevent transfer to the conceptus.

Uteroferrin has been demonstrated to cross the placenta (Chen et al., 1975) and be sequestered temporarily in the allantoic fluid compartment (Bazer et al., 1975). It was postulated that this was the principal mode of uteroferrin-mediated iron transport, absorption of iron from allantoic fluid. Absorption of uteroferrin by the allantoic epithelium does occur (Chapter VI). However, based on data presented in Chapters III and V, it seems that a great deal of uteroferrin storage/metabolism occurs at the placental level. Large increases in concentration as well as total uteroferrin content of the placenta were observed between days 45 and 75 of gestation. Beyond this time, levels remained fairly high. Endometrial culture data (Basha et al., 1979) revealed a decline in uterine production of uteroferrin beyond day 60. The relatively high placental levels after day 60 must, therefore, represent storage. Allantoic fluid uteroferrin/iron may represent "spillover"

from the placenta, i.e., uteroferrin not stored in placental tissue. Data from Chapter V support this concept. Twenty-four hours after injection of ^{59}Fe into the maternal circulation, high levels of radioactivity were present in fetal tissues (Figure 5.2), while it was difficult to demonstrate the presence of ^{59}Fe in unconcentrated allantoic fluid. Although the possibility of another protein involved in iron transport exists, uteroferrin appears to be the major iron transport protein secreted by the uterus during midgestation. Uterine secretions from unilaterally pregnant gilts injected with ^{59}Fe (Figure 5.3) demonstrated the presence of radiolabeled uteroferrin while levels of labeled transferrin were relatively small. Earlier studies by Palludan et al. (1969) demonstrated that iron transport in swine occurs at the level of the uterine glands and the areolae associated with them. It was further demonstrated that iron was transported in a nondialysable form, indicative of protein mediated transport. Results from earlier studies (Hoskins and Hansard, 1964a; Palludan et al., 1969) as well as data from this present study do not explain the factors involved in control of iron transfer.

It is interesting that the pig, with a more "primitive" placental type, does not appear to rely on transferrin for maternal to fetal transport of iron. Roberts and Bazer (1980) have speculated that there may be an evolutionary relationship between uteroferrin and transferrin. Both proteins have fairly similar absorption spectra, and the

amino acid composition of uteroferrin is similar to the carboxy terminal half of transferrin (Chen et al., 1973; Evans and Williams, 1978). Resonance Raman scattering also reveals similarities between uteroferrin and transferrin in the environment around the iron atom in each protein (Gaber et al., 1979). Another interesting observation is that a uteroferrin-like molecule has been observed in the horse (Zavy et al., 1979), which also has an epitheliochorial type of placentation.

In summary, uteroferrin appears to be intimately involved in iron transport in swine. A temporal relationship has been established between iron content and uteroferrin accumulation. The exact mode of uptake of uteroferrin bound iron is subject to further investigation.

APPENDIX A
ACID PHOSPHATASE, PROTEIN AND UTEROFERRIN ASSAYS

Lowry Protein Assay

Prepare enough Lowry C
    Mix Lowry A + B 50:1

Standard curve
    Blk, 20, 50, 100, 150, 200 400µg of BSA
    Bring to 1ml with water

Prepare samples
    Add correct aliquot
    Bring to 1ml with water

Add 5ml of Lowry C to each tube.
Site at r.t. at least 10 minutes.
Add 0.5ml of Phenol Reagent to each tube, vortexing as
    it's added.
Sit at r.t. 30 minutes.
Read at 750 nm with filter

Acid Phosphatase

Prepare: All on Ice

1. Buffers
   A. 40ml 0.2M HAc
      60ml 0.2M Na Acetate
      100ml H_2O

   B. 0.1264 gm Sigma 104
      60ml Buffer A
      0.12ml $MgCl_2$ Stock

2. Standard Curve
   Blk, 2, 5, 10, 15, 20, 25, 30, 40µl of P-nitrophenol
   Add 1ml Buffer B to each

3. Samples
   Make dilutions with Buffer A
   Bring to 1ml with Buffer B

Incubate 10 minutes at 30°.
Immediately add 1ml 0.25 n NaOh to each then 2mls more to each.
Read 410 nm.

Protocol for Uteroferrin RIA

Buffers:

Stock 0.2m sodium barbital
(41.2g/l)

Barbital buffer 200ml 0.2M stock
36 ml 0.2N HCl
0.58 g EDTA
dilute to 2000 ml
pH 8.0

Assay buffer 40ml BSA/1000ml
barbital buffer (.25% BSA-barbital)

1° antibody--use at dilution of 1/8000 for ~55% binding.

Standards

.143ml to 1ml with assay buffer = 100µg/ml. Dilute to 1/10 to yield 10µg/ml. Dilute 1/10 to yield 1µg/ml.

Dilutions

.05	µg = .05 (1µg/ml)	→ 1.0ml
.075	µg = .075(1µg/ml)	→ 1.0ml
.10	µg = .10 (1µg/ml)	→ 1.0ml
.25	µg = .25 (1µg/ml)	→ 1.0ml
.50	µg = .50 (1µg/ml)	→ 1.0ml
.75	µg = .75 (1µg/ml)	→ 1.0ml
1.0	µg = .10(10µg/ml)	→ 1.0ml
2.0	µg = .20(10µg/ml)	→ 1.0ml
4.0	µg = .40(10µg/ml)	→ 1.0ml
6.0	µg = .60(10µg/ml)	→ 1.0ml
8.0	µg = .80ml (10/µgml)	→ 1.0ml

0.1ml sample, standard or buffer
0.1ml 1° antibody
0.1ml ^{125}I-uteroferrin
0.5ml assay buffer
Incubate at 4 C for 72 h
Nonspecific binding, total count and blank tubes were also run.
After 72 h
0.1ml sheep γ globulin (400µg/ml)
0.1ml 2° antibody (rabbit antisheep γ globulin, 1 → 7)
Incubate at 4 C overnight

Centrifuge at 3000 rpm, 4 C for 30 min.
Wash with cold barbital buffer (2.0ml).
Centrifuge at 3000 rpm, 4C for 30 min.
Pour off supernatant, blot and count.

Iron and Molecular Weight Determination of Uteroferrin (UF)

Purified UF samples (2.790mg/ml) were processed as previously described for allantoic fluid. Iron concentration was determined by atomic absorption spectrophotometry. Values represent means ± standard errors.

% iron	.168 ± .007
approximate molecular weight	33950 ± 784

Minimal Essential Medium (MEM) Ingredients Per Liter

L-Arginine	70 mg	Hypoxanthine	.3 mg
L-Histidine	20 mg	Thymine	.3 mg
L-Lysine	70 mg	Uracil	.3 mg
L-Tyrosine	40 mg	Thiamine	.01 mg
DL-Tryptophane	20 mg	Riboflavin	.01 mg
DL-Phenylalanine	50 mg	Pyridoxine	.025 mg
L-Cystine	20 mg	Pyridoxal	.025 mg
DL-Methionine	30 mg	Niacin	.025 mg
DL-Serine	50 mg	Niacinamide	.025 mg
DL-Threonine	60 mg	Pantothenate	.01 mg
DL-Leucine	120 mg	Biotin	.01 mg
DL-Isoleucine	40 mg	Folic acid	.01 mg
DL-Valine	50 mg	Choline	.5 mg
DL-Gluatmic acid	150 mg	Inositol	.05 mg
DL-Aspartic acid	60 mg	p-Aminobenzoic acid	.05 mg
DL-Alanine	50 mg	Vitamin A	.1 mg
L-Proline	40 mg	Calciferol	.1 mg
L-Hydroxyproline	10 mg	Menadione	.01 mg
Glycine	50 mg	a-Tocopherol phosphate	.01 mg
L-Cysteine	.1 mg	Ascorbic acid	.05 mg
Adenine	10 mg	Glutathione	.05 mg
Guanine	.3 mg	Cholesterol	.2 mg
Xanthine	.3 mg		

L-Glutamine	100 mg
Adenosine triphosphate	1 mg
Adenylic acid	.2 mg
Ribose	.5 mg
Desoxyribose	.5 mg
Bacto-dextrose	1 g
Tween 80	5 mg
Sodium acetate	50 mg
Iron (ferric nitrate)	.1 mg
Sodium chloride	8 g
Potassium chloride	.4 g
Calcium chloride	.14 g
Magnesium sulfate	.2 g
Disodium phosphate	60 mg
Monopotassium phosphate	60 mg
Sodium bicarbonate	.35 g
Bacto-phenol red	20 mg
Triple distilled water	1000 ml

APPENDIX B
LEAST SQUARES PREDICTION EQUATIONS AND R^2 VALUES

PREDICTION EQUATIONS AND R^2 VALUES BASED ON LEAST SQUARES REGRESSION ANALYSIS FOR HEMOGLOBIN LEVELS AND ^{59}Fe ACCUMULATION IN FETAL BLOOD DURING GESTATION

Source	Estimated regression line	R^2
Hemoglobin	$y = 1.06+0.16\chi-0.01\chi^2$	.2718
^{59}Fe		
Whole blood	$y = 11630.85-188.49\chi+0.84\chi^2$	.4596
Plasma	$y = 887.89-13.89\chi+0.06\chi^2$	.1979
Cells	$y = 3245.06-54.99\chi+0.28\chi^2$	.1571

PREDICTION EQUATIONS AND R^2 VALUES BASED ON LEAST SQUARES REGRESSION ANALYSIS FOR ^{59}Fe ACCUMULATION BY FETAL TISSUES DURING GESTATION

Source	Estimated regression line	R^2
^{59}Fe concentration		
Liver	$y = -19696.29+1336.47\chi-19.27\chi^2+0.08\chi^3$	.4787
Spleen	$y = -476.95+210.43\chi-1.42\chi^2$	.0462
Kidney	$y = 1196.84-19.59\chi+0.09\chi^2$	.6807
Placenta	$y = 574.64-8.69\chi+0.45\chi^2$	.2984
Bone	$y = -4571.51+124.86\chi-0.68\chi^2$	.5441
Total ^{59}Fe		
Liver	$y = -73606.89+2654.51\chi-1.46\chi^2-0.11\chi^3$	.7267
Spleen	$y = -30837.73+759.66\chi-3.97\chi^2$	.7376
Kidney	$y = 64.92+2.20\chi+0.02\chi^2$	.3313
Placenta	$y = -33450.68+1873.99\chi-12.43\chi^2$	.3134

PREDICTION EQUATIONS AND R^2 VALUES BASED ON LEAST SQUARES REGRESSION ANALYSIS FOR IRON CONTENT OF FETAL TISSUES AND FLUIDS DURING GESTATION

Source	Estimated regression line	R^2
Iron concentration		
Liver	$y = 6453.68-295.02\chi+5.40\chi^2-0.04\chi^3+8.58\chi^4$	.3929
Fetus minus liver	$y = 1330.21-37.09\chi+0.26\chi^2+0.01\chi^3-1.48\chi^4$	.5164
Placenta	$y = -1164.38+104.32\chi-2.59\chi^2+0.03\chi^3$	.2366
Allantoic fluid	$y = -10.00+0.58\chi-0.01\chi^2+2.79\chi^3$	.1610
Amniotic fluid	$y = 197.77-10.61\chi+0.21\chi^2-0.01\chi^3$	.0613
Total iron		
Liver	$y = 572.13-39.49\chi+0.41\chi^2+0.13\chi^3$	.7484
Fetus minus liver	$y = 7.59-0.44\chi+0.01\chi^2-3.41\chi^3+1.25\chi^4$	.8513
Placenta	$y = -15206.96+1003.89\chi-23.47\chi^2+0.26\chi^3$	.5139
Allantoic fluid	$y = 3073.68-263.21\chi+8.23\chi^2-0.09\chi^3$	.2663
Amniotic fluid	$y = 31752.16-1860.18\chi+38.83\chi^2-0.33\chi^3$	.2058

PREDICTION EQUATIONS AND R^2 VALUES BASED ON LEAST SQUARES REGRESSION ANALYSIS FOR PLACENTAL UTEROFERRIN CONTENT DRUING GESTATION

Source	Estimated regression line	R^2
Uteroferrin concentration		
Placenta	$y = 7388.29-4249.24\chi+87.74\chi^2-0.76\chi^3+0.01\chi^4$	.5116
Total uteroferrin		
Placenta	$y = 12323.95-730.19\chi+15.42\chi^2-0.13\chi^3+0.01\chi^4$	.4731

PREDICTION EQUATIONS AND R^2 VALUES BASED ON LEAST SQUARES REGRESSION ANALYSIS FOR FETAL TISSUE WEIGHTS AND FLUID VOLUMES DURING GESTATION

Source	Estimated regression line	R^2
Liver	$y = 71.37-5.42\chi+0.14\chi^2-0.01\chi^3+5.46\chi^4$	.9188
Fetus minus liver	$y = 560.81-36.85\chi+0.76\chi^2-0.01\chi^3+1.76\chi^4$	.9475
Spleen	$y = -3.56+0.09\chi$	.8289
Kidney	$y = -8.53+0.2\chi-0.01\chi^2$	.7558
Placenta	$y = 585.69-50.89\chi+1.50\chi^2-0.02\chi^3+5.99\chi^4$	.6396
Allantoic fluid	$y = 1286.78-83.99\chi+2.22\chi^2-0.02\chi^3+8.88\chi^4$	.2783
Amniotic fluid	$y = 113.19-24.23\chi+0.83\chi^2-0.01\chi^3+2.81\chi^4$	.5468

PREDICTION EQUATIONS AND R^2 VALUES BASED ON LEAST SQUARES REGRESSION ANALYSIS FOR ACID PHOSPHATASE ACTIVITY IN ALLANTOIC FLUID AND PLACENTAL TISSUE DURING GESTATION

Source	Estimated regression line	R^2
Acid phosphatase concentration		
Allantoic fluid	$y = 28130.55-2124.01\chi+54.51\chi^2-0.55\chi^3$	.1573
Placenta	$y = 3445.68-165.64\chi+2.99\chi^2-0.02\chi^3+6.96\chi^4$	.2379
Total acid phosphatase		
Allantoic fluid	$y = 3103803.27-258989.13\chi+7319.93\chi^2-80.57\chi^3+0.30\chi^4$	.1116
Placenta	$y = -184280.31+10129.01\chi-184.92\chi^2+1.43\chi^3$	.0288

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Professor of Animal Science

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